(54) Title: BONE PRECURSOR CELLS: COMPOSITIONS AND METHODS

(57) Abstract

Disclosed are compositions of bone pre-
cursor cells and methods for their preparation
and use. Bone precursor cells are cells which
are not hematopoietic and which can differen-
tiate into osteoblasts upon exposure to a bone
growth factor and deposit calcium into the ex-
tracellular matrix. Such bone precursor cells are
useful in the treatment of certain bone related
disorders and diseases, such as osteoporosis, or
in promoting fracture repair. In addition, meth-
ods of differentiating bone precursor cells into
osteoblasts, and other diagnostic and even prog-
nostic methods are provided.

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DESCRIPTION
BONE PRECURSOR CELLS: COMPOSITIONS AND METHODS

SPECIFICATION

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The present application is a continuation-in-part of
co-pending U.S. Patent Application Serial No. 08/289,794,
filed August 12, 1994, the entire text and figures of
which disclosure is specifically incorporated herein by
10   reference without disclaimer. The U.S. Government may
own rights in the present invention pursuant to grant
numbers PO1-AG-08777 and 43460.

Field of the Invention

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This invention relates generally to methods for
obtaining bone precursor cells and compositions
comprising such cells. The invention includes methods
for enriching the population of bone precursor cells in
20   bone marrow cells isolated from mammalian bones or
peripheral blood. Also provided are methods for
differentiating bone precursor cells into osteoblasts,
and diagnostic and even prognostic methods.

25   **BACKGROUND OF THE INVENTION**

The rate of bone fractures in the United States is
estimated at 6,000,000 individuals per year. In 1984
(Holbrock et al., 1984) these injuries resulted in a
30   direct cost (i.e., excluding loss of income) of
$17,000,000,000 per year. When a bone is completely
fractured, a significant number of fractures require
medical intervention beyond simple immobilization
(casting), particularly those involving trauma. A major
35   problem in such instances is the lack of proximity of the
two bone ends (referred to as non-union). This results

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in an inappropriate and prolonged repair process, which may prevent recovery.

5   The average length of time for the body to repair a fracture is 25-100 days, for moderate load-bearing, and one year for complete repair. Thus, both simple fractures and medically complicated breaks would benefit from novel therapeutic modalities which accelerate and/or complete the repair process. The same is true for those
10   bone diseases (referred to as osteopenias) which result in a thinning of the bone the primary symptom of which is an often-debilitating fracture.

         Primary Osteoporosis is an increased, progressive
15   bone loss which accompanies the aging process. As such, it represents significant health risk in the United States which greater than 15 million Americans suffering from primary (idiopathic) osteoporosis resulting in a direct cost of $6,000,000,000 per year (Holbrock et al.,
20   1984). Primary osteoporosis is the most common of the metabolic bone diseases, and some 40,000-50,000 fracture-related deaths per year are attributed to this disorder. This mortality rate is greater than deaths due to cancer of the breast and uterus, combined. Significantly, this
25   disorder, which is one of the osteopenias, is asymptomatic until a bone fracture occurs. Affected individuals typically fracture the radius, femoral head, or collapse vertebrae.

30   Osteoporosis has a greater impact on the female population with larger numbers of women than men struck by this disorder, and a significant increase in the rate of osteoporosis occurs post-menopause. The rate of osteoporosis in these women is slowed but not ameliorated
35   by estrogen replacement therapy. Indeed, there is no convincing medical evidence that any treatment is successful in restoring lost bone mass of any kind.

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Given the aging of the American population, patients with osteoporosis also represent a significant target population for effective and novel bone therapies.

5 The process of aging in general is associated with a progressive diminution of bone-accumulation capacity, especially in trabecular bone (Nimni et al., 1993). This decreased structural integrity is associated with a number of alterations in bone proteins, osteoid
10 formation, calcium loss etc., leading to osteopenia (Nimni et al., 1993; Fedarko et al., 1992; Termine, 1990). The exact cellular mechanisms underlying such changes in bone structure and function are unclear. However, central to all of these alterations are cells of
15 the osteoblast lineage.

Reductions in osteoblast function or numbers, of necessity, leads to the loss of bone-forming capacity. It is known that some aspects of osteoblast function
20 decrease greatly with age (Termine, 1990). Overall, total protein synthesis and the synthesis of specific proteoglycans decreases markedly (Fedarko et al., 1992), whereas collagen and other proteins such as fibronectin and thrombospondin are degraded (Termine, 1990).
25

Bone cells from older individuals, in vitro, have the capacity to respond to growth factors, but their synthetic and proliferative capacity is diminished (Termine, 1990), presumably due to reduced responsiveness
30 to various osteogenic growth factors (Pfeilschifter et al., 1993). This results in diminished bone precursor cell and osteoblast numbers (Nimni et al., 1993).

There is no current treatment for lost bone mass,
35 including various growth-promoting proteins and Vitamin D_3. Likewise, there is no effective replacement or implant for non-union fractures or crush injuries of

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the bone. Currently, these latter types of injury
utilize bovine (cow), or human cadaver bone which is
chemically treated (to remove proteins) in order to
prevent rejection. However, such bone implants, while
5   mechanically important, are biologically dead (they do
not contain bone-forming cells, growth factors, or other
regulatory proteins). Thus, they do not greatly modulate
the repair process. All of these concerns demonstrate a
great need for new or novel forms of bone therapy.
10

        Bone development results from the proliferation of
mesenchymal cells, their differentiation into osteogenic
progenitor cells, and the eventual calcification of
cartilage and bone extracellular matrix (Urist et al.,
15   1983). Human bone marrow contains a distinct cell
population that expresses bone proteins and responds to
growth factor $\beta 1$ (TGF-β), but not to hematopoietic growth
factors (Long et al., 1990).

20   Little information exists concerning the growth
factors or cytokines controlling development of bone
precursor cells (osteoprogenitor cells and
preosteoblasts) into their differentiated progeny, the
osteoblasts. Likewise, few studies address the impact of
25   extracellular matrix (ECM) molecules on this stage of
human bone cell development, or the impact of aging on
either of these two areas. In the past, human bone cells
(both precursor cells and osteoblasts) have been
technically difficult to acquire and
30   purification/characterization studies or protocols were
few in number. Additionally, current in vitro models of
bone formation are limited as the use of post-fetal
mesenchymal tissue to generate bone cells often results
in chondrogenesis, but is inadequate for osteogenesis,
35   (Urist et al., 1983). Thus, information concerning the
cellular activation signals, differentiation, and bone

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matrix production during the early phases of human bone
cell development is limited, at best.

5   The regulation of chondro-osteogenic gene activation
is induced during bone morphogenesis by an accumulation
of extracellular and intracellular signals (Urist et al.,
1983). Importantly, extracellular signals are known to
be transferred from both cytokines and extracellular
matrix molecules (Urist et al., 1983), to responding cell
10   surface receptor(s) resulting in eventual bone formation.
The formation of bone occurs by two mechanisms. Direct
development of bone from mesenchymal cells (referred to
as intramembranous ossification; as observed in skull
formation) occurs when mesenchymal cells directly
15   differentiate into bone tissue. The second type of bone
formation (the endochondral bone formation of skeletal
bone) occurs via an intervening cartilage model.

The development and growth of long bones thus
20   results from the proliferation of mesenchymal cells,
their differentiation into osteogenic progenitor cells
and (then) osteoblasts, cartilage deposition, and
eventual calcification of the cartilage and/or bone
matrix. Concurrently, bone is remodeled to form a
25   tubular bone space in which hematopoietic cell
differentiation occurs.

Interestingly, the number of osteoprogenitor cells
in adult bone seems too small to replace all of the large
30   mass of bone normally remodeled in the process of aging
of skeleton (Urist et al., 1983). Further observations
(vide infra) confirm this concept by showing that one
(unexpected) source of osteoprogenitor cells is the bone
marrow. This reduced progenitor cell number also implies
35   that there is a disassociation of bone progenitor cell
recruitment from subsequent osteogenic activation and

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bone deposition, and further suggests multiple levels of
regulation in this process.

        One of the central issues concerning bone formation
5   regards the developmental lineages of the bone cell
     types, namely the osteoblast and the osteoclast. There
     is adequate evidence to suggest that osteoblasts arise
     from local mesenchymal cell populations, and that
     osteoclasts are derived from blood-born
10   monocyte/macrophage cells.

        Fischman and Haye first demonstrated that monocytes
     fused to form osteoclasts in regenerating newt limbs
     (Fishman et al., 1962). Although the role of macrophage
15   fusion remains controversial (Hattersley et al., 1989 and
     Horton et al., 1985), further evidence for the blood-born
     origin of the osteoclast was pioneered by LeDouarin using
     a chick:quail chimera in which nuclear morphology allows
     clear distinction of cell derivation. These studies
20   conclusively demonstrated that osteoblasts and osteocytes
     are derived from the limb bud mesenchyma whereas
     osteoclasts arise from blood-born hematopoietic cells
     (Jotereau et al., 1978 and Le Douarin, 1973). The
     importance of these observations was subsequently shown
25   by the successful cure (by osteoclasts) of osteopetrosis
     utilizing bone marrow transplantation in both animals
     (Ash et al., 1980), and humans (Coccia et al., 1980).
     While such data conclusively show the hematogenous origin
     of the osteoclast, little knowledge exists on the nature
30   or location of the stem cell population(s) capable of
     differentiating into bone-forming osteoblasts.

        Like other developing tissues, bone responds to
     bone-specific, and other soluble growth factors. TGF-β is
35   a member of a family of polypeptide growth regulators
     that affect cell growth and differentiation during
     developmental processes, such as embryogenesis and tissue

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repair (Sporn *et al.*, 1985). TGF-*β* strongly inhibits
proliferation of normal and tumor-derived epithelial
cells, blocks adipogenesis, myogenesis, and hematopoiesis
(Sporn *et al.*, 1985). However, in bone, TGF-*β* is a
5   positive regulator.

TGF-*β* is localized in active centers of bone
differentiation (cartilage canals and osteocytes)
(Massague, 1987), and TGF-*β* is found in high quantity in
10   bone - suggesting that bone contains the greatest total
amount of TGF-*β* (Massague, 1987 and Gehron Robey *et al.*,
1987). During bone formation, TGF-*β* also promotes
chondrogenesis (Massague, 1987) - an effect presumably
related to its ability to stimulate the deposition of
15   extracellular matrix (ECM) components (Ignotz *et al.*,
1986). Besides stimulating cartilage formation, TGF-*β* is
synthesized and secreted in bone cell cultures, and
stimulates the growth of sub-confluent layers of fetal
bovine bone cells, thus showing it to be an autocrine
20   regulator of bone cell development (Sporn *et al.*, 1985).

In addition to TGF-*β*, other growth factors or
cytokines are implicated in bone development. Urist and
co-workers have been able to isolate various regulatory
25   proteins that function in both *in vivo* and *in vitro*
models (Urist *et al.*, 1983). Bone morphogenic protein
(BMP), originally an extract of demineralized human bone
matrix, has now been cloned (Wozney *et al.*, 1988), and
when implanted *in vivo* results in a sequence of events
30   leading to functional bone formation (Wozney *et al.*, 1988
and Muthukumaran *et al.*, 1985). The implanting of BMP is
followed by mesenchymal cell migration to the area of the
implant, differentiation into bone progenitor cells,
deposition of new bone, and subsequent bone remodeling to
35   allow the establishment of bone marrow (Muthukumaran *et
al.*, 1985).

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A number of additional growth factors exist which
regulate bone development. In particular, bone-derived
growth factors (BDGF) stimulate bone cells to proliferate
in serum-free media (Hanamura *et al.*, 1980 and Linkhart
5   *et al.*, 1986). However, these factors seem to function
at a different level from BMP (Urist *et al.*, 1983).

The extracellular matris (ECM) varies in its tissue
composition throughout the body, consisting of various
10   components such as collagen, proteoglycan, and
glycoprotein (Wicha *et al.*, 1982). Numerous studies
point to the influences of ECM in promoting cellular
development. Gospodarowicz *et al.* demonstrated that ECM,
the natural substrate surrounding cells *in vivo*, greatly
15   affects corneal epithelial cell proliferation *in vitro*
(Gospodarowicz and Ill, 1980 and Gospodarowicz *et al.*,
1980). Studies by Reh *et al.* (1987) show that
extracellular components such as laminin are involved in
inductive interactions which give rise to retinal and
20   retinal pigmented endothelium. Also, differentiation and
growth of mammary epithelial cells are profoundly
influenced by ECM components, and mammary cell growth *in
vivo* and *in vitro* requires type IV collagen (Wicha *et
al.*, 1982), Finally, studies from one of the inventor's
25   laboratories show that bone marrow ECM also plays a major
role in hematopoiesis in that complex ECM extracts
greatly augment cell proliferation (Campbell *et al.*,
1985), and that marrow-derived ECM contains specific
cytoadhesion molecules (Campbell *et al.*, 1987; Campbell
30   *et al.*, 1990; Long and Dixit, 1990; Long *et al.*, 1990;
and Long *et al.*, 1992).

A number of non-collagenous matrix proteins,
isolated from demineralized bone, are involved in bone
35   formation. Osteonectin is a 32 kDa protein which,
binding to calcium, hydroxyapatite and collagen, is
believed to initiate nucleation during the mineral phase

of bone deposition (Termine *et al.*, 1981). *In vivo*
analysis of osteonectin message reveals its presence in a
variety of developing tissues (Nomura *et al.*, 1988 and
Holland *et al.*, 1987). However, it is present in its
5   highest levels in bones of the axial skeleton, skull, and
the blood platelet (megakaryocyte) (Nomura *et al.*, 1988).

Bone gla-protein (BGP, osteocalcin) is a vitamin K-
dependent, 5700 Da calcium binding bone protein that is
10   specific for bone and may regulate Ca++ deposition
(Termine *et al.*, 1981; Price *et al.*, 1976; and Price *et
al.*, 1981). Other bone proteins seem to function as
cytoadhesion molecules (Oldberg *et al.*, 1981 and Somerman
et al., 1987), or have unresolved functions (Reddi,
15   1981).

While bone morphogenesis is ECM dependent, bone ECM
also contains a number of the more common mesenchymal
growth factors such as PDGF, basic, and acidic fibroblast
20   growth factor (Urist *et al.*, 1983; Linkhart *et al.*, 1986;
Hauschka *et al.*, 1986; and Canalis *et al.*, 1985). These
activities are capable of stimulating the proliferation
of mesenchymal target cells (BALB/c 3T3 fibroblasts,
capillary endothelial cells, and rat fetal osteoblasts).
25   As well, bone-specific proliferating activities such as
the BMP exist in bone ECM.

While these general and specific growth factors
undoubtedly play a role in bone formation, little is
30   understood concerning the direct inductive/permissive
capacity of bone-ECM or bone proteins themselves on human
bone cells or their progenitors. Nor is the role of bone
ECM in presenting growth factors understood - such
"matricrine" (factor:ECM) interactions may be of
35   fundamental importance in bone cell development but have
not been well characterized.

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SUMMARY OF THE INVENTION

The present invention provides the isolation,
purification and characterization of precursors to
5   osteoblasts, and the identification of human
osteoprogenitor cells. Immunological separation of bone
marrow non-adherent low-density (NALD) cells results in a
marked enrichment of bone precursor cells that express
osteocalcin, osteonectin, and bone alkaline phosphatase.
10

The bone precursor cells of the present invention,
although isolatable from bone marrow, are not part of the
bone marrow stromal cell compartment, nor are they a
component of the hematopoietic cell lineages. The lack
15   of a stromal cell nature is demonstrated by the failure
to isolate these cells from human stromal cell isolates,
and physical cell separation by density centrifugation.
These cells are not hematopoietic as demonstrated by
their failure to express the pan-hematopoietic cell
20   antigen CD34, and their failure to respond to
hematopoietic growth factors.

In addition to other distinguishing features, the
present invention is distinct from the prior art in that
25   the prior art studies are generally confined to
osteogenic cultures in which bone cells are observed in
bone marrow-derived stromal cell populations (Gronthos *et
al.*, 1994; Friedenstein *et al.*, 1987; Luria *et al.*, 1987;
Turksen and Aubin, 1991; Van Vasselaer *et al.*, 1994).
30   Given the combined physical and immunological separation
disclosed herein, the present population of bone
precursor cells likely represents an earlier stage of
bone precursors than the prior art, in that the present
immune-isolated cells are not intimately associated with
35   the endosteal surface of the bone marrow trabeculae.

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Flow cytometric analyses show that distinct cell
subpopulations exist among these isolated cells. The
majority of the bone Protein, antigen-positive cells are
preosteoblasts, approximately the size of a lymphocyte,
5   whereas other, antibody-separated subpopulations consist
of osteoblasts and osteoprogenitor cells. In serum-free
cultures, TGF-β stimulates the small, antigen-positive
cells to become osteoblasts as these cells both increase
in size, cellular complexity, and express increased
10   levels of osteocalcin and alkaline phosphatase.

Antibody-separated cells also contain a separate
population of progenitor cells that form colonies of
osteoblast cells when cultured in serum-free, semi-solid
15   media. Two types of these osteoprogenitor cells are
observed: a colony-forming cell (CFC) that generates
several hundred bone antigen-positive cells, and a more
mature cluster-forming cell that has a lesser
proliferative potential and thus generates clusters of
20   20-50 antigen-positive cells.

Osteopoietic colony-forming cells and cluster-
forming cells have an obligate, but differential
requirement for osteogenic growth factors. The CFCs
25   respond to TGF-β, basic fibroblast growth factor (bFGF),
bone morphogenetic protein-2 (BMP-2), and 1, 25-dihydroxy
vitamin D3 (1,25-OH D3). In contrast to the colony-
forming cells, cluster-forming cells are regulated
predominately by 1,25-OH D3 and TGF-β, but fail to
30   respond to bFGF.

The inventors thus defined that human bone marrow
contains a non-hematogenous, heterogeneous population of
bone precursor cells among which exists a population of
35   proliferating osteoprogenitor cells. The present
provision of these bone precursor cell populations in

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sufficient numbers allows evaluation of their role in
osteogenesis in both health and disease.

5   In one aspect, the present invention provides a
process for preparing an enriched population of bone
precursor cells. The process generally comprises the
steps of:

        (a)   obtaining a population of cells that include
10   bone precursor cells;

        (b)   enriching the population for bone precursor
              cells by exposing the population of cells to a
              bone precursor cell antibody immunoreactive
15   with a bone precursor cell antigen; and

        (c)   removing cells of the population that do not
              immunoreact with a bone precursor cells
              antibody.

20   The population of cells that includes bone precursor
cells may be a population of bone marrow cells, a
population of cells isolated from bone, or a population
of peripheral blood cells.

25   Bone precursor cells can be further enriched by
equilibrium-density centrifugation of the population of
bone marrow or peripheral blood cells. Equilibrium-
density centrifugation of the cell population provides
30   low density bone marrow cells enriched in bone precursor
cells with a density of between about 1.050 and about
1.090 gm/cm^3, preferably between 1.060 and 1.085 gm/cm^3.

        In another aspect, stromal cells present, e.g., in
35   bone marrow cells, can be removed by exposing bone marrow
cells to an adherent surface, typically tissue culture
plastic or tissue culture glass.

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In yet another aspect, an enriched population of bone precursor cells is further fractionated according to size. In one embodiment, size fractionation can be accomplished by fluorescence activated flow cytometry,
5 velocity sedimentation, or counter-flow centrifugal elutriation. Bone precursor cells of the present invention generally have average diameters of between about 8 microns and about 70 microns, and preferably, of between about 10 microns and about 20 microns.
10

Antibodies are used to enrich the population of bone precursor cells. Suitable antibodies include any antibody immunoreactive with a bone precursor sell. Bone precursor cell antibodies particularly contemplated by
15 the present invention include anti-osteocalcin, anti-osteonectin, and anti-bone alkaline phosphatase.

Physico-chemical separation techniques, such as equilibrium density centrifugation, can be used to obtain
20 a moderate enrichment of bone precursor cells, e.g., to a level of about 6-7% purity. Density separation and plastic adherence are used to further increase the purity of such cells.

25 A significant contribution of the present invention is the use of immunoseparation techniques to obtaining substantially purified populations. The use of immuneadherence separation generates substantially pure populations of human bone precursor cells. As used
30 herein, the term "substantially pure" refers to a population of bone precursor cells that is between about 60% and about 80% pure. Immuno-magnetic separation, preferably using anti-osteonectin and anti-osteocalcin antibodies, yields an almost homogeneous or essentially
35 pure population of bone precursor cells. The term "essentially pure", as used herein, refers to a

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population of bone precursor cells that is about 95%
pure.

5   In using a second antibody immunoreactive with a
bone precursor cell antibody, enhanced enrichment of the
population of bone precursor cells is thus achieved.   In
one embodiment, antibodies are conjugated to a solid
substrate including: tissue culture plastic, agarose,
other plastics, polyacrylamide, or magnetic particles.

10

The present invention thus provides a population of
bone precursor cells enriched about 100-fold or more over
the starting materials, i.e., over the bone marrow cells
or peripheral blood cells that include bone precursor

15   cells.   More preferably, the population of bone precursor
cells is enriched between about 1,000-fold and about
2,000-fold, or between about 2,000-fold and about 3,000-
fold, or between about 3,000-fold and about 4,000-fold,
over the starting cell population, with enrichment of up

20   to about 4,800-fold being achievable.

In one embodiment, mammalian bone precursor cells
are contemplated by the present invention.   In a
preferred embodiment, bone precursor cells from human

25   bone marrow cells are contemplated.

The present invention further provides a composition
comprising bone precursor cells.   Bone precursor cells as
provided herein generally have the following

30   characteristics:

        (a)   immunoreactive with a bone precursor cell
               antibody;

35   (b)   average cell diameter of 8 microns to about 70
               microns; and

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(c)   differentiate into osteoblasts upon exposure to
       tissue growth factor β, 1,25-OH Vitamin D3,
       basic fibroblast growth factor, or bone
       morphogenic protein 2.

5

In one aspect, the composition comprising bone
precursor cells can be prepared as described above from
mammalian bone, bone marrow, or peripheral blood cells.
Bone precursor cells of the present invention include
10   cells immunoreactive anti-osteocalcin, anti-osteonectin
or anti-bone alkaline phosphatase. In one embodiment,
bone precursor cells express osteocalcin, osteonectin or
alkaline phosphatase but do not express the pan-
hematopoietic antigen CD34. In a preferred embodiment,
15   bone precursor cells include osteoprogenitor cells and
preosteoblasts.

In yet another aspect, a method of differentiating a
bone precursor cell into an osteoblast is provided by the
20   present invention. The method generally comprises the
steps of:

(a)   obtaining a population of bone precursor cells
       according to the procedure described above;

25

(b)   exposing the bone precursor cell to a growth
       factor; and

(c)   cultivating the bone precursor cell under serum
30   free conditions to differentiate the bone
       precursor cell into an osteoblast.

Growth factors contemplated include transforming
growth factor β, insulin-like growth factor (IGF) and
35   platelet-derived growth factor (AA, A/B, and B/B
isoforms) 1,25-OH Vitamin D3, basic fibroblast growth
factor, or bone morphogenic protein. In one embodiment,

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a bone precursor cell is exposed to a single growth
factor. Alternatively, a bone precursor cell can be
exposed to two or more growth factors.

5   In another embodiment, the method of differentiating
a bone precursor cell into an osteoblast further
comprises cultivating the bone precursor cell in the
presence type I collagen, fibrinogen, fibrin,
vitronectin, thrombospondin, osteocalcin, or osteonectin.
10   In one embodiment, bone precursor cells are cultivated in
the presence of type I collagen, fibrinogen and fibrin.
In an alternative embodiment, bone precursor cells are
cultivated in the presence of type I collagen,
fibrinogen, fibrin, vitronectin, thrombospondin,
15   osteocalcin, and osteonectin.

         The present invention further provides diagnostic
and prognostic methods. In certain embodiments, the
invention therefore includes methods for identifying a
20   subject at risk of developing an age-related bone
disorder, which methods generally comprise the steps of:

         (a)   obtaining a population of cells from the
               subject, the population being enriched for
25   human bone precursor cells; and

         (b)   quantifying the amount of a bone precursor
               related protein, such as, e.g., osteocalcin or
               osteonectin, expressed by the bone precursor
30   cells, wherein an increased or otherwise
               altered amount of the protein, in comparison to
               the amount within the bone precursor cells of a
               young or middle-aged subject, is indicative of
               a subject at risk of developing an age-related
35   bone disorder, such as osteoporosis.

A currently preferred example of this method includes the steps of:

(a) obtaining a population of cells from the subject, the population being enriched for human bone precursor cells; and

(b) quantifying the amount of osteocalcin or osteonectin expressed by the bone precursor cells, wherein an increased amount of osteocalcin or osteonectin, in comparison to the amount within the bone precursor cells of a young or middle-aged subject, is indicative of a subject at risk of developing an age-related bone disorder, such as osteoporosis.

These methods are generally based on the finding that osteonectin and osteocalcin antigenic expression by human preosteoblast cells increases with increasing age in a statistically significant manner. Osteonectin expression is particularly elevated in older subjects (an increase from 59 to 89 arbitrary log units).

Further methods of the invention include the diagnosis of particular groups or sub-sets of elderly subjects that have, or are at risk of developing, a certain type of bone disease or disorder, particularly osteoporosis or osteopenias or another of the group of bone disorders connected with increased aging. These methods generally comprise:

(a) obtaining a population of cells from the elderly subject, the population being enriched for human bone precursor cells; and

(b) quantifying the amount of osteocalcin or osteonectin expressed by the bone precursor

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cells, wherein a decreased amount of
osteocalcin or osteonectin, in comparison to
the average amount within the bone precursor
cells of an elderly subject, is indicative of
5   an elderly subject having a particular type of
osteoporosis, osteopenia or age-related change
in bone formation.

Decreased amounts of osteocalcin and osteonectin,
10   and most particularly, decreased amounts of osteonectin,
in elderly subjects have been discovered to be indicative
of an elderly subject having a particular type of
osteoporosis, osteopenia or other disorder associated
with age-related changes in bone formation, such as those
15   individuals having a more severe form of osteoporosis.

This is based upon the inventors' findings that the
majority of bone precursor cells from a certain sub-set
of elderly subjects belonged to an antigen-dull
20   population. As elderly subjects with bone disorders can
generally be characterized into two main groups, and as
the methods of the invention generally allow two main
types of bone precursor cells to be identified (one of
which is the antigen-dull population), the diagnostic
25   utility of the invention in distinguishing between these
two groups is evident.

In any of the diagnostic or prognostic methods, the
composition comprising the bone precursor cells will
30   generally be prepared as described above and may be
obtained from human a bone marrow or peripheral blood
sample. The enrichment steps of the cell preparation
method will preferably provide for a significantly
purified human bone precursor cell population, will more
35   preferably include an immunomagnetic separation step, and
will most preferably include immunomagnetic separation

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using anti-osteocalcin and/or anti-osteonectin
antibodies.

5   The most preferred method of quantifying the amount
       of osteocalcin or osteonectin expression is to use
       fluorescence activated flow cytometry. However, the use
       of other immunological methods, such as RIAs, ELISAs, and
       the like, is certainly contemplated; as is the use of
       molecular biological methods based upon the hybridization
10   of DNA segments, probes or primers comprising osteocalcin
       or osteonectin sequences.

                        BRIEF DESCRIPTION OF THE FIGURES

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       The following drawings form part of the present
       specification and are included to further demonstrate
       certain aspects of the present invention. The invention
       may be better understood by reference to one or more of
20   these drawings in combination with the detailed
       description of specific embodiments presented herein.

       FIG. 1 in four panels shows flow cytometric analysis
       of immune-adherent bone marrow derived bone precursor
25   cells. The circled area represents larger osteoblast
       cells. FIG. 1A shows cells which express osteocalcin.
       FIG. 1B shows cells that express bone alkaline
       phosphatase. FIG. 1C shows the differentiation of bone
       precursor cells that express osteocalcin into
30   osteoblasts. FIG. 1D shows the differentiation of bone
       precursor cells that express bone alkaline phosphatase
       into osteoblasts.

       FIG. 2 in two panels shows the In Vitro Expansion of
35   TGF-β stimulated bone marrow derived osteoblasts.
       FIG. 2A shows the increase in the frequency of
       osteoblasts following 7 days of culture in the presence

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of TGF-β. FIG. 2B shows the increase in the total number
of osteoblasts following 7 days of culture in the
presence of TGF-β.

5   FIG. 3 in two panels shows two photographs of human
bone marrow derived osteoprogenitor colonies. FIG. 3A is
a photograph of progeny of osteoprogenitor cluster-
forming cells. FIG. 3B is a photograph of progeny of
osteoprogenitor colony-forming cells.

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       FIG. 4 in two panels shows the responsiveness of
colony-forming and cluster-forming osteoprogenitor cells
to various growth factors. FIG. 4A shows the effect of
the labelled growth factors on cluster-forming
15   osteoprogenitor cells. FIG. 4B shows the effect of the
labelled growth factors on colony-forming osteoprogenitor
cells.

       FIG. 5 in three panels shows the immunophenotypic
20   characterization of human preosteoblast and osteoblast
cells. Human bone precursor cells were isolated by
immune-adherence or immunomagnetic separation. For back-
gating, antigen positive cells were electronically marked
(identified by square regions in the figure) and antigen
25   negative or dull cells similarly marked (as identified by
circles in the figure; upper sub-panels, right and left).
These marked populations were then displayed to show
their forward angle and side-scatter characteristics.
       FIG. 5A in four sub-panels shows forward angle and
30   side-scatter characteristics of un-induced and
TGF-β-induced human bone precursor cells isolated by
immune-adherence. Upper sub-panels, day 0 (un-
induced) immune adherent cells and differentiated
cells following 7 days of TGF-β stimulation (left
35   and right sub-panels, respectively). Horizontal
dashed lines represent upper limit of non-specific
(inappropriate antibody) fluorescence, vertical

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lines represent the upper 95% size limit for input
NALD cells, both as defined herein and in Long *et
al.* (1995). Lower sub-panels, back-gating of
antigenically marked cells to display their forward
5   and side scatter characteristics.

FIG. 5B in two panels shows forward and side-scatter
characteristics of immunomagnetically purified human
bone precursor cells. Immunomagnetically separated
10   cells were subjected to two color analysis of
osteonectin and osteocalcin expression and back-
gated. Dashed lines represent upper limit of non-
specific (inappropriate antibody) fluorescence as
described above, and determined by single-color
15   analysis of each fluorescent signal, both as defined
herein and in Long *et al.* (1995).

FIG. 5C in two panels shows bone protein expression
and phenotypic analysis of human bone precursor
20   cells from a subpopulation of elderly individuals.
Among the 17 individuals age $\geq$ 60 yrs. old, three
females (aged 60, 88, and 89 yrs old) and one male
showed a distinctly different preosteoblasts
antigenic profile. These individuals show a
25   predominant population of cells that are
antigenically dull (left panel). However, back
gating of these cells demonstrates that they have
similar forward and side-scatter characteristics to
human bone precursor cells from their age-matched
30   cohort, but lack high numbers of ON^{++} and OC^{++}
bright cells. A single individual (age 89) from the
group is shown.

FIG. 6 in two panels shows co-expression of
35   osteonectin and osteocalcin by purified populations of
human bone precursor cells. Human bone precursor cells
were purified by combined physico-chemical separation

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(density and plastic-adherence) and immunomagnetic
separation, and then simultaneously incubated with
antibodies to osteonectin and osteocalcin. FIG. 6A shows
that the first two physical separation steps results in a
5   moderate enrichment of bone-antigen-positive cells.
FIG. 6B shows that the immunomagnetic separation results
in an essentially antigenically pure cell population that
co-express osteonectin and osteocalcin. FIG. 6A and
FIG. 6B are divided into four quadrants that are numbered
10   and referred to in the text.

FIG. 7 in two panels shows expression of osteonectin
and osteocalcin in younger and older individuals.
FIG. 7A shows purified human bone precursor cells
15   evaluated for osteocalcin. FIG. 7B shows purified human
bone precursor cells evaluated for osteonectin. Bone
protein expression by human preosteoblast cells from a
single representative young individual (9 years old) is
indicated by the open profile and a representative older
20   (60 yrs) individual by the shaded profile. These
individuals were chosen as representative because their
mean-specific, and peak-fluorescence values were
identical to the average determined for their age-
cohorts, and their coefficients of variation were
25   similar.

FIG. 8 in a single panel shows age-related
alterations in mean specific fluorescence of osteonectin
and osteocalcin. Single color antigenic profiles of
30   purified human bone precursor cells populations were used
to determine mean specific fluorescence. $* = p \leq 0.05$,
Student's t-test, two-tailed.

FIG. 9 in a single panel shows age-related changes
35   in bone protein expression. The mean specific
fluorescence was plotted against the mean age for each
group young ($\leq$ 25 years old, mean age 16.4 $\pm$ 7 (S.D.)

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years, range 1.5 - 24 years, n = 15; middle age
individuals (mean age 36.6 ± 5 years old, range 26-45
years old, n=9), and elderly (≥ 50 years old, mean age
68.8 ± 7 years, range 53-89 years, n=13). Circles,
5   Osteocalcin (BGP); Inverted triangles, Osteonectin.

        FIG. 10 in two panels shows the labeling and
adhesion of human bone precursor cells to extracellular
matrix proteins and cytokines. Human bone precursor
10   cells were purified by immunomagnetic separation as
described in FIG. 6A and FIG. 6B. The cells were then
labeled with a "caged" fluorochromes (calcein, an
acetylmethyl ester derivative of fluroisothiocyanate).
FIG. 10A shows a standard curve of the linear
15   relationship between fluorescent intensity and cell
number. FIG. 10B shows the attachment of human bone
precursor cells, purified by immunomagnetic separation,
that were labeled with calcein, and adhered to plastic-
immobilized proteins. The percent attachment was
20   determined by reading the fluorescent signal of attached
cells on the standard curve in FIG. 10A.

DETAILED DESCRIPTION OF THE INVENTION

25   The present invention provides a process for
preparing an enriched population of bone precursor cells.
The process generally comprises the steps of:

        (a)   obtaining a population of cells that include
30   bone precursor cells;

        (b)   enriching the population for bone precursor
              cells by exposing the population of cells to a
              bone precursor cell antibody immunoreactive
35   with a bone precursor cell antigen; and

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     (c)   removing cells of the population that do not
           immunoreact with a bone precursor cells
           antibody.

5   In one embodiment, mammalian bone precursor cells
are contemplated by the present invention. In a
preferred embodiment, bone precursor cells from human
bone marrow cells are contemplated.

10   In certain embodiments, bone precursor cells may be
obtained from a population of bone marrow cells. In
other embodiments, bone precursor cells may be obtained
from a population of peripheral blood cells.

15   As used herein, a bone precursor cell is any cell
that is capable of differentiating or expanding into an
osteoblast cell. A bone precursor cell of the present
invention is not hematopoietic and thus does not express
the pan-hematopoietic antigen CD34. Preferred bone
20   precursor cells include osteoprogenitor cells of both the
colony forming cell type and the cluster forming cell
type and preosteoblast cells. As described in Example 1,
colony forming osteoprogenitor cells are antecedent to
cluster forming osteo-progenitor cells, and to
25   preosteoblast cells in the differentiation process.

      Bone precursor cells can be further enriched by
equilibrium-density centrifugation of the starting cell
population. Equilibrium-density centrifugation of such
30   cells provides low density cells enriched in bone
precursor cells with a density of between about 1.050 and
about 1.090 gm/cm^3.

      In a preferred embodiment, the density of bone
35   precursor cells is between about 1.060 and about 1.085
gm/cm^3. In one embodiment, equilibrium-density
centrifugation can be performed before the antibody

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purification of step (b) above. In this embodiment, the
antibody purification step is carried out on bone marrow
cells with a density of between about 1.050 and about
1.090. In a second embodiment, equilibrium-density

5   centrifugation can be performed after the antibody
purification of step (b) above. Alternatively, the
equilibrium-density centrifugation purification step can
be performed twice - once before the antibody
purification of step (b) above, and once after the

10   antibody purification step.

In another aspect, the population of bone precursor
cells can be enriched by removing stromal cells present,
e.g., in bone marrow cells. Removal of stromal cells can

15   be accomplished by exposing, e.g., bone marrow cells, to
an adherent surface, typically tissue culture plastic or
glass. Stromal cells adhere to tissue culture plastic or
glass while bone precursor cells do not. Stromal cells
can be removed before or after the immune purification

20   step. Preferably, stromal cells are removed prior to the
immune purification step. The use of solid surfaces such
as tissue culture plastic or glass is well known in the
art. Tissue culture plastic and glass can be treated
(e.g. silicone, nitrocellulose, nickel, etc.) to promote

25   or inhibit cell adhesion. Treated and untreated surfaces
are available commercially.

In another aspect, an enriched population of bone
precursor cells is further fractionated according to

30   size. In a preferred embodiment, size fractionation can
be accomplished by fluorescence activated flow cytometry.
Bone precursor cells of the present invention have
average diameters of between about 8 microns and about 70
microns. Preferably, bone precursor cells have average

35   diameters of between about 10 microns and about 20
microns.

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Notably, the use of multi-parameter flow cytometry shows that there are two size populations among immune adherent cells, and TGF-β-induced differentiation results in a shift between the small and large cell compartment.

5   In order to further examine the developmental characteristics of these cells, isolated uninduced and induced cells were electronically divided into small and large cell compartments and "back-gated" to determine the side-scatter characteristics of each. For back-gating,

10   antigen-positive, or negative cells are electronically marked and these marked populations re-analyzed to show their forward angle (i.e., cell size) and side-scatter (i.e., cell content) characteristics).

15   These data show that the larger osteoblast cells also have the highest degree of intracellular organization (side-scatter characteristics). This was seen in both uninduced cells (in which osteoblast-sized cells represent approximately 5% of the isolate) and

20   induced cells. Thus, the TGF-β-induced differentiation of human bone precursor cells results in an increase in antigenic content, cell size, and an increase in intracellular complexity. Isolated cells also contain a population of cells in which antigen density is little

25   different from non-specific antibody controls. Back-gating of this population consistently shows these cells as having a low degree of side-scatter. These characteristics (i.e., low side-scatter and small size) are consistent with a residual population of lymphoid-

30   like which do not bear detectable bone antigens.

The present invention provides an enriched population of bone precursor cells from about 100-fold over the starting material of bone marrow or peripheral

35   blood cells that include the bone precursor cells. Enrichment of between about 1,000-fold and about 2,000-fold, between about 2,000-fold and about 3,000-fold,

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between about 3,000-fold and about 4,000-fold over the
original bone marrow or peripheral blood cells is
possible, with enrichment of up to about 4,800-fold being
described herein.

5

Bone precursor cells of the present invention are
immunoreactive with bone precursor cell antibody. A bone
precursor cell antibody is used to enrich the population
of bone precursor cells. Bone precursor cell antibodies

10   contemplated by the present invention include anti-
osteocalcin, anti-osteonectin, and anti-bone alkaline
phosphatase. Anti-osteocalcin, anti-osteonectin, and
anti-bone alkaline phosphatase are described in Shull et.
al., 1984; incorporated herein by reference. As bone

15   precursor cells are further characterized, other
antibodies which immunoreact with a bone precursor cell
may be generated by one of ordinary skill in the art.
The use of these other antibodies immunoreactive with a
bone precursor cell is contemplated.

20

In a preferred embodiment, a bone precursor cell
antibody is conjugated to a solid substrate. The solid
substrate is preferably a tissue culture or petri dish.
The use of solid surfaces such as tissue culture plastic

25   or glass is well known in the art. Tissue culture
plastic and glass can be treated (e.g. silicone,
nitrocellulose, nickel, etc.) to promote or inhibit
protein adhesion. Treated and untreated surfaces are
available commercially.

30

As discussed in detail in Example 1, antibody coated
tissue culture dishes are utilized to "pan" for bone
precursor cells. Briefly, bone marrow cells containing
bone precursor cells are incubated on antibody coated

35   dishes. Bone precursor cells adhere to the antibodies
while all other cells do not adhere to the dish. After
incubation, the dish non-adherent cells are removed by

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gently washing the dish with media. Bone precursor cells
are removed from the dish and further analyzed, purified
or differentiated into osteoblasts.

5   In another embodiment, a second antibody
immunoreactive with a bone precursor cell antibody can be
used to enrich the population of bone precursor cells.
The use of a secondary antibody is generally known in the
art. Typically, secondary antibodies are antibodies
10   immunoreactive with the constant regions of the first
antibody. Preferred secondary antibodies include anti-
rabbit, anti-mouse, anti-rat, anti-goat, and anti-horse
and are available commercially.

15   In a preferred embodiment, secondary antibodies are
conjugated to a solid substrate including tissue culture
dish, agarose, polyacrylamide, and magnetic particles.
In this embodiment, a bone precursor cell antibody is
first immunoreacted to a bone precursor cell. The bone
20   precursor cell with the attached antibody is next exposed
to the secondary antibody that is conjugated to a solid
substrate. Enrichment of precursor cells is achieved
because only cells that present a bone precursor cell
antibody immunoreact with the secondary antibody. A
25   commercially available kit provides secondary antibodies
conjugated to magnetic particles. In this system, bone
precursor cells that present a bone precursor cell
antibody are purified by exposure to a magnetic field.

30   Although physico-chemical separation (equilibrium
density centrifugation) results in a moderate enrichment
of bone precursor cells to a level of about 6-7% purity,
and density separation followed by plastic adherence
further increases the purity, immunoseparation techniques
35   are particularly preferred for obtaining substantially
purified populations. The use of immuneadherence
separation generates substantially pure (~60-80%)

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populations of human bone precursor cells. Immuno-
magnetic separation based on the osteonectin and
osteocalcin antigens yields an almost homogeneous, i.e.,
about 95% pure, population of cells. This represents an
5   approximate 4,800-fold purification over unfractionated
bone marrow.

Immunomagnetically separated cells were subjected to
two-color fluorescence-activated cytometry to examine the
10   expression of osteonectin and osteocalcin. These data
show that the isolated cells co-express both proteins.
Moreover, antigen-density contour plots demonstrate that
these antigens are co-expressed in a single population of
cells, in that no distinct sub-populations of single-
15   antigen positive cells are detected. There remains,
however, a small population of antigen-dull cells.

Unlike the antigenically null-population seen in
cells separated by immune-adherence, the magnetically
20   separated antigen-low or dull cells have the same side-
scatter characteristics as the double-positive cell
population. Given that these cells are recovered
following two passes through the magnetic isolation
column, it is unlikely that these are contaminating
25   lymphoid cells (as are seen in immune adherence-based
separation). Rather, they represent cells with a
sufficient (albeit low) antigen density (albeit a low
density) to retain them on the column in the presence of
a magnetic field.
30

The preparation of bone precursor cell antibodies
was reported in Shull et al., 1989, incorporated herein
by reference. Both polyclonal and monoclonal antibodies
are contemplated by the present invention. Means for
35   preparing and characterizing antibodies are well known in
the art (See, e.g., Antibodies "A Laboratory Manual, E.
Harlow and D. Lane, Cold Spring Harbor Laboratory, 1988).

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Briefly, a polyclonal antibody is prepared by
immunizing an animal with an immunogen comprising a
polypeptide of the present invention and collecting
antisera from that immunized animal. A wide range of
5   animal species can be used for the production of
antisera. Typically an animal used for production of
anti-antisera is a rabbit, a mouse, a rat, a hamster, a
sheep or a guinea pig. Because of the ease of use and
relatively large blood volume of rabbits, a rabbit is a
10   preferred choice for production of polyclonal antibodies.

A monoclonal antibody can be readily prepared
through use of well-known techniques such as those
exemplified in U.S. Patent No. 4,196,265, herein
15   incorporated by reference.

Typically, a technique involves first immunizing a
suitable animal with a selected antigen (e.g.,
osteocalcin, osteonectin or bone alkaline phosphatase) in
20   a manner sufficient to provide an immune response. After
a sufficient time to induce an immune response, spleen
cells from the immunized animal are then fused with cells
of an immortal myeloma cell. A number of immortal
myeloma cells are available and the choice of the
25   immortal cell is within the skill of an artisan.
Immortal myeloma cells lack the salvage pathway of
synthesizing nucleotides.

The fused spleen/myeloma cells are cultured in a
30   selective medium to select fused spleen/myeloma cells
from the parental cells. Fused cells are separated from
the mixture of non-fused parental cells by the addition
of agents that block the *de novo* synthesis of nucleotides
in the tissue culture media. Unfused myeloma cells lack
35   the enzymes necessary to synthesize nucleotides from the
salvage pathway and are selectively killed in the
selective media. Unfused lymphocytes also do not

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continue to grow in tissue culture. Thus, only cells
that have successfully fused (hybridoma cells) can grow
in the selection media. The hybridoma cell produces a
monoclonal antibody.

5

The present invention further provides a composition
comprising bone precursor cells. Bone precursor cells as
provided herein have the following characteristics:

10   (a)   immunoreactive with a bone precursor cell
              antibody;

        (b)   average cell diameter of about 8 microns to
              about 70 microns; and

15

        (c)   differentiate into osteoblasts upon exposure to
              tissue growth factor β, 1,25-OH Vitamin D3,
              basic fibroblast growth factor, or bone
              morphogenic protein.

20

In addition, bone precursor cells have low side
scatter (right-angle light scatter) characteristics by
flow cytometry.

25   In one embodiment, the composition comprising bone
precursor cells can be prepared as described above from
mammalian bone marrow cells. Bone precursor cells
include cells immunoreactive anti-osteocalcin, anti-
osteonectin or anti-bone alkaline phosphatase. In one
30   embodiment, bone precursor cells express osteocalcin,
osteonectin or alkaline phosphatase but do not express
the pan-hematopoietic antigen CD34. In a preferred
embodiment, bone precursor cells include osteoprogenitor
cells or preosteoblasts.

35

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The differentiation cascade of bone precursor cells into an osteoblast is as follows:

Colony forming cell → Cluster forming cell →
5 *Preosteoblast → Osteoblast.*

Osteoprogenitor cells are herein defined as cells that proliferate to yield differentiated bone cells (preosteoblasts or osteoblasts) as their progeny. The
10 osteoprogeniter cells are of two types: a colony forming cell or a cluster-forming cell. A cluster forming cell is a progenitor cell with limited proliferative potential. As discussed in Example 1, a cluster forming cell differentiates into a colony of 20-50 bone-protein
15 antigen-positive (e.g. osteocalcin) cells after about 7 days of incubation.

A colony forming cell is a progenitor cell with increased proliferative potential. After around 7 days
20 of incubation, a colony forming cell differentiates into several hundred intensely bone antigen-positive cells.

A preosteoblast is a cell that differentiates into an osteoblast.
25

It is understood that these developmental stages cannot be defined precisely. As used herein, a bone precursor cell is any cell that is capable of differentiating or expanding into an osteoblast. A bone
30 precursor cell of the present invention includes osteoprogenitor cells, colony forming cells, cluster forming cells and preosteoblast cells.

Although not the only means of definition, human
35 bone preosteoblast cells may be characterized as small lymphocyte-sized cells with low side-scatter characteristics (SSC). Human preosteoblast cells may

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also be characterized as OC^{lo}, ON^{lo}, AP^{lo}, $CD34^-$, SSC^{lo}, and FAS^{lo}. Human osteoblast cells may be characterized as OC^{++}, ON^{++}, AP^{++}, $CD34^-$, SSC^{hi}, FAS^{hi}.

5   In yet another aspect, a method of differentiating a bone precursor cell into an osteoblast is provided by the present invention. The method generally comprises the steps of:

10   (a)   obtaining a population of bone precursor cells according to the procedure described above;

        (b)   exposing the bone precursor cell to a growth factor; and

15

        (c)   cultivating the bone precursor cell under serum free conditions to differentiate the bone precursor cell into an osteoblast.

20   Growth factors used include transforming growth factor β, 1,25-OH Vitamin D3, basic fibroblast growth factor, or bone morphogenic proteins. In one embodiment, a bone precursor cell is exposed to a single growth factor. Alternatively, a bone precursor cell can be
25   exposed to two or more growth factors.

        Upon exposure to a growth factor, the bone precursor cell differentiates into an osteoblast. Culturing the precursor cell for seven days in the presence of a growth
30   factor causes the cell to enlarge approximately 3-fold (FIG. 1A, FIG. 1B, FIG. 1C, FIG. 1D). There is also a 5-fold increase in the antigenic content of the cell. Furthermore, the total number of osteoblasts increase approximately 4-5 fold (FIG. 2). These results are
35   discussed in greater detail in Example 1.

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In still another embodiment, the method of
differentiating a bone precursor cell into an osteoblast
further comprises cultivating the bone precursor cell in
the presence of type I collagen, fibrinogen, fibrin,
5   polyglycolic acid, polylactic acid, osteocalcin, or
osteonectin. In one embodiment, bone precursor cells are
cultivated in the presence of type I collagen,
fibrinogen, and fibrin. In one embodiment, type I
collagen, fibrinogen, fibrin, polyglycolic acid,
10   polylactic acid, osteocalcin, or osteonectin is used
alone in the presence of a growth factor. In an
alternative embodiment, bone precursor cells are
cultivated in the presence of type I collagen,
fibrinogen, fibrin, osteocalcin, and osteonectin. It is
15   understood that any combination of the compounds listed
above in this paragraph is contemplated by the present
invention.

Bone precursor cells cultivated in the presence of
20   type I collagen, fibrinogen, fibrin, osteocalcin, and
osteonectin produced an extracellular matrix similar to
bone. These cultures were positive in the Von Kossa
reaction and were capable of depositing calcium ino the
extracellular matrix. Bone precursor cells cultivated in
25   a collagen/fibrin matrix are useful in therapeutic
applications.

This invention provides a cellular product of
isolated, purified animal bone precursor cells, and the
30   means to expand these bone cells as a transplantation
therapy for osteopenias. In this modality, an animal's
own bone precursor cells, or those of a histocompatible
donor, can be removed and if necessary, expanded in
culture. Next, these cells or an *ex vivo*, expanded
35   number of these bone-forming cells, are transplanted into
animals with bone formation deficits. The use of an
autologous animal bone precursor cell transplant would

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avoid graft rejection. Autologous or allogeneic bone
cell transplantation will be of therapeutic importance in
the treatment of osteopenias. In the majority of these
disorders, the tightly linked balance of bone formation
5   and bone reabsorption is disrupted, with reabsorption
being predominant. The infusion of large numbers of
bone-forming cells will thus augment bone formation in
diseases such as osteoporosis.

10   Purified animal bone precursor cells are also an
ideal target for animal gene therapy for bone disorders,
in which a known, specific molecular defect results in
abnormal bone formation. The ability to isolate and
stimulate the division of bone precursor cells
15   demonstrates that they can be used as target cells for
gene therapy of congenital bone defects.

Another use of animal bone precursor cells is a
cell-based biological matrix which is implanted in
20   animals requiring any form of bone repair or bone union.
This product is comprised of bone cells imbedded within a
biologically active protein matrix. The biomatrix
consists of defined bone proteins (e.g. type I collagen,
osteonectin, fibrinogen, fibrin and osteocalcin), and
25   specific bone growth factors (above) which stimulate bone
cell proliferation. Alternatively, polyglycolic acid
and/or polylactic acid can also be present. The bio-
matrix acquires its consistency (that of a firm gel) and
shape by the activation of fibrinogen as a protein
30   polymer and the subsequent gel transition of collagen.

The cells used in this wholly biological implant are
the animal's own bone precursor cells (i.e. the cells
are autologous and therefore not rejected, see above)
35   which are isolated, and (if needed) expanded in tissue
culture. This expansion results in a population of early
bone-forming cells which is then embedded within the bio-

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matrix by polymerization of the gel protein. The result
is a "pre-bone" implant containing the animal's own bone
cells which would rapidly reorganize the implant and
mineralize it. Moreover, the gel-phase will allow this
5   bone substitute to be formed in any necessary shape, thus
facilitating small-bone replacement, its use in
periodontal disease, or cosmetic repair.

Bone precursor cells can be obtained by a variety of
10   immunologically-based procedures. These are comprised
of, but not limited to, fluorescence-activated flow
cytometry, immunological-based column chromatography,
antibody-conjugated sepharose beads (or other inert
beads), or other immunology based applications (e.g
15   immuno-magnetic separation). These procedures do not
however, define the population of animal bone precursor
cells, but rather lead to its isolation. Other physical
separation procedures may be applied prior or after the
antigenic purification. These are comprised of, but not
20   limited to, equilibrium density centrifugation, velocity
sedimentation, or counter-flow centrifugal elutriation.

As well, other antigenic markers may be used to
positively or negatively further define these cells.
25   These are comprised of, but not limited to, antigens of
the animal major histocompatibility locus (particularly
HLA-DRA), hematopoietic antigens (e.g., CD33, CD8, CD10,
CD14, CD9, CD20), or other bone proteins.

30   The animal bone precursor cells described herein are
isolated from animal bone marrow. Sources of such marrow
are the flat bones of the axial skeleton (ribs, hips,
sternum), as well as the humeri, radi, ulanea, tibulae,
and fibulae. Additionally, these cells can be obtained
35   from other non-marrow sources including, but not limited
to, the periosteum, bone trabeculae, cancellous bone, or
the endosteum.

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Furthermore, the present inventors have shown that animal bone precursor cells circulate, thus making it possible to recover and purify these cells in animal peripheral blood. Indeed, human bone precursor cells
5   have now been isolated from human peripheral blood and shown to have similar flow cytometry characteristics to those of bone marrow.

The present invention further provides a method of
10   identifying/screening novel bone growth factors. Candidate bone growth factors or cytokines are screened using an enriched population of bone precursor cells. Bone precursor cells are stimulated to differentiate in the presence of a bone growth factor. Newly identified
15   bone growth factors or cytokines are used in the treatment of osteopenias, fractures and could also be used in the aforementioned bone cell bio-matrix implants.

In another embodiment, bone precursor cells can be
20   used to treat *osteogenesis imperfecta*. *Osteogenesis imperfecta* is a disease with an identified genetic defect. In this embodiment, bone precursor cells from a patient with *Osteogenesis imperfecta* are isolated. A copy of the defective gene without the mutation is
25   introduced into the bone precursor cell through well known transfection/transformation techniques. Bone precursor cells containing the corrected gene are reintroduced into the patient or are first expanded *in vitro* and then reintroduced into the patient. This
30   procedure can be performed as a heterologous or autologous procedure. A preferred embodiment is through an autologous procedure.

In yet further embodiments, the present invention
35   provides diagnostic methods that may be used to evaluate age-related alterations in human bone precursor cells by assessing bone protein expression. These methods were

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developed following a series of flow cytometric
investigations on immunomagnetically-separated bone
precursor cells, which demonstrated that age-related
changes in bone protein expression occur with increasing
5   age (see, e.g., Example 4).

The studies were performed on a total of
41 individuals of three age groups: $\leq$ 25 years old (mean
age 16.4 $\pm$ 7 (S.D.) years, range 1.5-24 years, n=15),
10   50 years old; (mean age 36.6 $\pm$ 5 years old, range 26-45
years, n=9) and individuals $\geq$ 50 years old (mean age
70.1 $\pm$ 12 years, range 53-89 years, n=17).

Human bone precursor cells were isolated and
15   purified cells from individuals in the given age groups
and subjected to multi-parameter flow cytometric
analysis. As expected, antigenically-purified human bone
precursor cells from these three age populations co-
express both osteonectin and osteocalcin. Interestingly,
20   osteonectin and osteocalcin antigenic expression by human
preosteoblast cells increases with increasing age.

The flow cytometry data clearly illustrate that the
human bone precursor cells in older individuals (*i.e.*,
25   $\geq$ 50 years of age) express higher amounts of these two
bone proteins than do younger individuals (*i.e.*,
$\leq$ 25 years old). Profiles from middle aged individuals
were intermediate to the other two age groups.

30   In order to determine whether these alterations were
statistically significant for the whole population, the
mean specific- and peak-fluorescence were determined for
each individual in each age group. A significant
(p $\leq$ 0.05) age-related increase was noted in both the
35   mean specific fluorescence for osteocalcin (BGP) and
osteonectin, as well as in the peak fluorescence of each
antigen. Osteocalcin shows a moderate but significant

change in mean fluorescence, increasing by 21 arbitrary
log units. In contrast, osteonectin expression increases
to a greater degree in the population of older
individuals (an increase from 59 to 89 arbitrary log
5   units).

     The inventors further analyzed this increase in bone
protein levels by examining the relationship between age
and antigenic expression. Although the numbers of
10   individuals of middle age are lower than the other two
age-cohorts, it nonetheless appears that the majority of
the increase in human bone precursor cell osteonectin and
osteocalcin levels occurs between the ages of 15-16
and 35-40.
15

     The observation that osteonectin and osteocalcin
expression in purified populations of human bone
preosteoblast cells increases with increasing age is
consistent with reports showing that serum osteocalcin
20   expression increases with age in both males and females
(Delmas et al., 1983; Orwoll and Deftos, 1990). The
inventors' observation demonstrates the cellular basis of
the reported serum increases in osteocalcin; i.e., the
bone precursor cells themselves express increased
25   osteocalcin (as well as osteonectin).

     The age-related increase in expression of
osteonectin in purified populations of human (non-
adherent) preosteoblasts found in this invention is in
30   contrast to that reported by Fedarko et al. (1992).
These investigators documented a progressive decrease in
osteonectin levels following 15 years of age. However,
comparisons of the methods of isolation between the
present report, and that of Fedarko's shows that
35   distinctly different cell populations were isolated.

- 40 -

The inventors' separation procedure isolates a population of cells by physico-chemical, and immunological means. In contrast, the Fedarko study examines preosteoblasts/osteoblasts which are
5   enzymatically (collagenase) released from mineralized matrix, and thus differ greatly from those in the present report. Therefore, the present invention and the Fedarako study likely concern cells at different stages of development. For example, early preosteoblasts not
10   intimately associated with endosteal surface may, when so adherent, respond to regulatory signals within the mineralized bone extracellular matrix which result in the down-regulation of expression/synthesis of specific bone proteins.
15

Of the seventeen individuals greater than 60 years of age evaluated, four (three women, age 64, 88; and 89 years old; one male age 59) did not show the human bone precursor cell immunophenotypic characteristics described
20   above. Rather, the majority (80-90%) of the presumed preosteoblasts isolated from these individuals are similar in antigenic content to the antigen-dull population of cells described above. These individuals thus lack the predominant osteocalcin-
25   positive/osteonectin-positive (OC^{++}/ON^{++}) bright population observed in all other individuals (n=37), including their age-related cohort (both male and female).

30   Given the immunomagnetic retention of these cells on the column, and that the forward-angle and side-scatter characteristics of these cells are identical to that of the antigen-dull cell subpopulation, the inventors conclude that these individuals possess a population of
35   preosteoblasts which differ greatly in their antigenic expression. Consistent with this, the mean and peak fluorescence of both osteonectin and osteocalcin are

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significantly reduced, even when compared to the youngest
age-cohort (Table 2).

5   These data suggest that a separate subpopulation of
individuals exists with distinctly different (lower)
human bone precursor cell antigenic content. This sub-
population of elderly people in which only a predominant
population of antigen^{dull} preosteoblast cells are present
could represent subjects with a differentiation blockade
10   or, alternatively, a loss (or masking) of antigen on the
preosteoblast.

Early observations suggest that these aspects of the
invention have diagnostic and, perhaps, prognostic
15   utility in connection with certain bone disorders. For
example, the use of multi-parameter flow cytometry and
immunophennotyping may allow the diagnosis and prediction
of outcomes (prognosis) of various bone disorders such as
primary osteoporosis. There are two types of primary
20   osteoporosis. Type 1 osteoporosis strikes females in the
first two decades following menopause, whereas Type 2
osteoporosis effects both males and females in their 6th
and 7th decade of life. The pattern of antigenic
expression in human bone precursor cells observed by the
25   inventors shows that elderly individuals ($\geq$ 60 years of
ago) are of two types: those with statistically high bone
antigen content, and those with significantly low antigen
content (compared to age-matched controls). This
suggests that these values may reflect the disease status
30   of the affected individual's bone function.

Thus, the immuno-phenotypic purification and multi-
parameter flow cytometry based characterization of human
bone precursor cells provides an important means of
35   defining the cellular and biochemical basis of both age-
related, and perhaps also, disease-mediated alterations
in bone cells and their precursors. Further, the

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invention can be used to monitor the outcome of the
transplantation of human bone precursor cells into
patients with bone formation deficits.

5   The following examples are included to demonstrate
preferred embodiments of the invention. It should be
appreciated by those of skill in the art that the
techniques disclosed in the examples that follow
represent techniques discovered by the inventors to
10   function well in the practice of the invention, and thus
can be considered to constitute preferred modes for its
practice. However, those of skill in the art should, in
light of the present disclosure, appreciate that many
changes can be made in the specific embodiments that are
15   disclosed and still obtain a like or similar result
without departing from the spirit and scope of the
invention.

Example 1:   Preparation of Bone Precursor Cells
20

Bone Marrow Cell Preparation and Culture. Human bone
marrow aspirates were obtained from normal volunteers.
Bone marrow cells were subjected to adherent cell
depletion, and density separation techniques as
25   previously described (Long *et al.*, 1988). Briefly, non-
adherent, low density (NALD) cells were prepared by first
subjecting the cells to equilibrium-density
centrifugation (Ficoll). The resultant mononuclear, low
density cells next were subjected to two rounds of
30   plastic adherence and these non-adherent, low density
(NALD) cells then subjected to immune-adherence isolation
(*vide infra*). Bone cells were cultured in supplemented
McCoy's 5A media (Long *et al.*, 1988) containing 1% ITS+
(Collaborative Research, Bedford, MA) as described
35   previously (Long *et al.*, 1990).

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Antibodies, Immune-Adherence, and Immunochemistry. The
expression of bone protein antigens was determined by
fluorescence activated flow cytometry, or by
immunocytochemistry utilizing a avidin-biotin system as
5   previously described (Long *et al.*, 1990; Long and
Heffner, 1988). These antibodies are specific for their
respective antigens and do not cross-react with other
matrix proteins (Stenner *et al.*, 1984 and Shull *et al.*,
1989). The monoclonal antibody to bone alkaline
10   phosphatase (SAOS2-P80) was raised to osteosarcoma cells
(Shull *et al.*, 1989). This antibody was proven to detect
alkaline phosphatase activity by immunoprecipitation, and
by direct protein sequence-analysis of the precipitated
antigen. For immune-adherence, monoclonal antibodies to
15   osteocalcin and osteonectin were immobilized onto tissue
culture plastic using a procedure described previously
(Long *et al.*, 1992). Approximately 2.5 - 6.5 x 10^5 NALD
cells per cm^2 were incubated on antibody-coated dishes
for one hour at 37° C. Subsequently, non-adherent cells
20   were removed with three rounds of gentle washing using
serum-free McCoy's media containing 1% BSA. The immune-
adherent cells were removed with the aid of a rubber
policeman and analyzed for flow cytometry (as input
cells) or cultured for seven days *in situ* in the presence
25   or absence of osteogenic growth factors.

Flow Cytometric Analysis. Flow cytometric analysis was
performed using a Becton-Dickinson FACSCAN system and
data analyzed with the BD Lysis software program.
30   Controls consisted of autofluorescence as well as non-
specific fluorescence detected with isotype-specific
murine monoclonal antibody to keyhole lympet hemocyanin
obtained from Becton Dickinson. Human osteoblast cells
lines (MG63, SAOS2,) were stained with monoclonal
35   antibodies to osteocalcin, and alkaline phosphatase,
respectively, and analyzed for fluorescence intensity
versus size (forward angle scatter) versus cellular

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complexity (side scatter) to independently map the
position of osteoblast cells in immune isolated bone
marrow cell populations. The frequency of osteoblast
cells defined as the number of cells occurring in the
5   osteoblast cell region (as defined above; circle in
FIG. 1A, FIG. 1B, FIG. 1C, FIG. 1D) is expressed as a
percent of total bone antigen positive cells.

Osteogenic Colony-Forming Cells. In order to quantify
10   osteoprogenitor cells in immune-adherent cultures,
adherent cells were cultured *in situ* in a fibrin-clot
system. Plasminogen-free fibrinogen (Hematologic
Technologies, Burlington VT; 2 mg/mL) was treated with
human thrombin (1 Unit/mL) to form a fibrin-clot. The
15   known capacity of osteoblasts to produce proteolytic
enzymes and thus lyse the clot was overcome by using
epsilon-amino caproic acid at a concentration of 5 mM,
and repletion with more fibrinogen, as needed.
Immunocytochemistry for osteocalcin expression was
20   performed as previously reported (Long *et al.*, 1990),
except that diaminobezadine concentration was 3 mg/mL and
the substrate incubation period was increased to 2 hours.
Immunocytochemistry controls consisted of an
inappropriate antibody, secondary antibody only, and
25   diaminobezadine only (to assess endogenous peroxidase
activity). These controls were uniformly negative.

*Isolation and Enrichment of Bone Protein Expressing
Cells.* A number of non-collagenous proteins play a role
30   in bone formation. Monoclonal antibodies were utilized
to two of these two bone proteins, osteocalcin and
osteonectin, both as phenotypic markers of bone cell
development, and as a mechanism of isolating bone
protein-expressing cells *in vitro*. Osteonectin (also
35   known as SPARC) is present in high concentration in bone
of the axial skeleton and skull (Nomura *et al.*, 1988 and
Holland *et al.*, 1987). Osteonectin binds to calcium,

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hydroxyapatite, and collagen and, thus, may regulate
mineral deposition in bone matrix (Termine *et al.*, 1981).
Another bone protein, osteocalcin (also known as bone
gla-protein or BGP) is a vitamin K-dependent protein that
5   is specific for bone, and also binds calcium (Termine *et
al.*, 1981; Hauschka *et al.*, 1975; Price *et al.*, 1976; and
Price *et al.*, 1981). The inventors also utilized a
monoclonal antibody to bone alkaline phosphatase as a
phenotypic marker for bone cells.
10

The inventors' previous data demonstrated that
marrow-derived osteoblasts modify extracellular matrix in
an osteogenic fashion (Long *et al.*, 1990). Coupled with
the present data on antigenic phenotyping and *in vitro*
15   expansion, the inventors herein define marrow-derived
preosteoblasts as small, proliferating cells (lymphocyte-
sized) that express low amounts of bone protein antigens,
whereas marrow-derived osteoblast cells are the larger,
differentiated progeny of these cells which express high
20   amounts of antigen and, with longer culture, generate an
osteoid matrix. FIG. 1A, FIG. 1B, FIG. 1C and FIG. 1D
shows the flow cytometric analysis of human non-adherent
low-density (NALD) bone marrow cells which were incubated
on antibody-coated tissue culture dishes. Immune-
25   adherent bone cell populations were analyzed for
expression of bone proteins as well as cell size
(forward-angle light scatter; FAS).

FIG. 1A shows osteocalcin positive cells and FIG. 1B
30   shows bone alkaline phosphatase positive cells. Antigen-
positive cells are identified as those cells with more
fluorescence than the upper limit of non-specific
fluorescence seen in antibody controls (shaded area).
Osteoblasts cells (circled) were identified as those bone
35   protein antigen-positive cells significantly larger than
the upper 95% cell size limit for input NALD cells

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(dashed line) as well as parallel analyses of human
osteoblasts.

        Monoclonal antibodies to osteocalcin and osteonectin
5   (Stenner *et al.*, 1984 and Shull *et al.*, 1989) were
    utilized to capture ("pan") bone protein-expressing NALD
    cells using conventional immune-adherence technology
    (Wysocki *et al.*, 1978 and Mage *et al.*, 1992). Immune-
    adherence isolation produces a markedly enriched
10 population of cells that are 40-60% bone-protein antigen-
    positive. Subpopulations of these cells were further
    resolved using multiparameter fluorescence-activated flow
    cytometry (FIG. 1A, FIG. 1B, FIG. 1C, FIG. 1D).

15   The first subpopulation comprises the majority of
    the cells and is distinguished as a group of bone
    antigen-positive preosteoblasts approximately the size of
    a lymphocyte. A second population of antigen-positive
    osteoblasts is larger than the upper 95% limit of
20 unfractionated NALD cell populations and comprises
    approximately 2-4% of the immune-adherent cell population
    (FIG. 1A and FIG. 1B). Both antigen-positive small and
    large cells express osteocalcin, bone alkaline
    phosphatase (upper right and left panels), and
25 osteonectin. The exact developmental level at which bone
    precursor cells express these antigens is unknown.
    However, two-color fluorescence cytometry studies
    indicate that the majority ($\geq$90%) of the immune-isolated
    cells co-express both osteonectin and BGP (*vide infra*).
30

    *In Vitro Expansion and Differentiation of Bone Marrow
    Derived Bone Cells.* Immune-isolated bone precursor cells
    were cultivated under serum-free conditions in the
    presence of TGF-β1. Multi-parameter flow cytometry shows
35 that TGF-β treatment causes the small cells to
    differentiate into large, bone-protein antigen-positive
    cells (FIG. 1C and FIG. 1D). Following seven days of

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serum-free culture, the majority of immune-isolated cells
show an approximate 3-fold increase in cell size (as
defined by forward-angle light scatter), and a coordinate
increase in their antigenic content, as demonstrated by a
5   0.5- to 1.0-log increase in relative fluorescence.

     FIG. 1C and FIG. 1D show the flow cytometric
analysis of bone precursor cells cultured for 7 days in
the presence of 25 pM TGF-β. FIG. 1C shows the
10   differentiation of osteocalcin positive cells into
osteoblasts. Fig. 1D shows the differentiation of bone
alkaline phosphatase cells into osteoblasts.

     This observation shows that unfractionated marrow
15   NALD cells contain bone precursor cells which, in long-
term, serum-replete cultures, acquired the morphological
and functional characteristics of osteoblasts (Long et
al., 1990). Thus, these osteoblasts produced bone
proteins, and deposited these proteins into the
20   extracellular matrix that, subsequentially, begins
mineralization (as indicated by cellular deposition of
calcium into the matrix and positive Von Kossa staining)
(Long et al., 1990).

25   Examination of such osteoblasts demonstrate that
their frequency increases approximately 4-5-fold in the
presence of TGF-β (FIG. 2A). This increased cell
frequency is due to increased proliferation of bone
protein antigen-positive cells. Examination of the total
30   cellularity per culture shows an approximate 3-4-fold in
vitro expansion in the total number of osteoblasts over a
7-day period (FIG. 2B), while the antigen-negative cell
number remains unchanged or diminished. This is true for
both BGP-expressing cells as well as alkaline
35   phosphatase-positive cells.

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It is highly unlikely that bone precursor cells die off in culture while the larger osteoblasts proliferate to become the predominant cell population because osteoblasts have low, if any, proliferative capacity.

5 Immune selection of bone protein antigen-positive cells yields a TGF-β-responsive population of small bone precursor cells capable of differentiating into osteoblasts.

10 *In Vitro Development of Human Osteoprogenitor Cells*. There exists among the immune-isolated bone precursor cells a true bone progenitor cell, i.e., precursor cells capable of undergoing clonal expansion into differentiated progeny. The clonal nature and *in vitro*

15 characteristics of progenitor cells in other systems are well described: progenitor cell growth and development requires the presence of at least one mitogenic growth factor, and cell growth in an inert, semi-solid 3-dimensional matrix, which results in the clonal

20 formation of cell colonies by restricting the outgrowth of differentiated progeny (Metcalf *et al.*, 1989). To detect osteoprogenitor cells, immune-isolated NALD cells were over-layered with chemically defined, serum-free media containing fibrinogen - which is subsequently

25 treated with thrombin to form a fibrin-clot. Cells were cultured for 7 days under serum-free conditions in the presence of TGF-β (or other growth factors *vide infra*). Subsequently, the fibrin clot was dried to a film, and the cultures subjected to immunocytochemical analysis.

30

Two types of progenitor cell-derived colonies are observed after 7 days. One colony phenotype consists of small clusters of cells containing 20-50 osteocalcin-positive cells (FIG. 3A). By convention, this type of

35 progenitor cell is referred to as a cluster-forming cell (Metcalf *et al.*, 1989 and Metcalf *et al.*, 1983) and represents a progenitor cell with limited proliferative

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potential.   Immune-adherent bone antigen-positive cells
were cultured in serum-free tissue culture media (Long *et
al.*, 1990) containing plasminogen-free fibrinogen, which
was treated with thrombin (Briddell *et al.*, 1990) to form
5   a fibrin-clot.   Following 7 days of culture, the fibrin
clots were dried to a film, and subjected to
immunocytochemical analysis using monoclonal antibodies
to human BGP (osteocalcin) as described elsewhere
(Stenner *et al.*, 1984).

10

The second type of osteogenic cell growth consists
of colonies containing several hundred intensely
osteocalcin-positive cells (FIG. 3B).   This latter type
of colony-forming cell (CFC) thus represents an
15   osteoprogenitor cell with an increased proliferative
potential.   Under appropriate growth factor conditions
(see below), this type of progenitor cell is present at
20-50 CFC per 10^5 total immune-adherent cells.   These two
types of progenitor cell growth are consistent with
20   previous observations in other systems in which colony-
forming cells are thought to be more primitive than
cluster-forming cells (Metcalf *et al.*, 1989 and Metcalf
et al., 1983).   Therefore, the cluster-forming cell
represents a later (more mature) stage of bone cell
25   development than the colony forming cell (i.e., the CFC-
colony is developmentally antecedent to the CFC-cluster).

Both cluster-forming, and colony-forming
osteoprogenitor cells show an obligate requirement for
30   growth factors, and a differential responsiveness to
bone-regulatory cytokines (FIG. 4A and FIG. 4B).   Both
progenitor cell types fail to develop in the absence
osteogenic growth factors (media controls, FIG. 4A and
FIG. 4B), whereas the addition of recombinant human
35   growth factors known to regulate osteoblasts (Urist *et
al.*, 1983; Hauschka *et al.*, 1986; Noda *et al.*, 1989;
Rodan *et al.*, 1989; and Wozney *et al.*, 1988) stimulates

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both cluster and colony formation. The colony-forming
progenitor cells respond equally well to TGF-β and bFGF,
generating approximately 40-60 colonies per 10^5 cells
(FIG. 4B). Likewise, 1,25-OH D3 and BMP-2 both stimulate
5   colony forming cells, but to a lesser degree than that
seen with TGF-β or bFGF.

The more mature cluster-forming osteoprogenitor
cells respond best to 1,25-OH vitamin D3 (vit. D3; values
10   are negative logarithms of molarity), intermediately well
to both bone morphogenic protein (BMP-2; values are
ng/mL) and transforming growth factor-β (TGF-β; values
are pM), but fail to respond to basic fibroblast growth
factor (bFGF, values are ng/mL) (FIG. 4A). Bars labeled
15   as "Media" are immune-adherent cells cultured in serum-
free conditions without the addition of exogenous growth
factors. Interestingly, the observation that bFGF fails
to drive the formation of osteogenic clusters suggests a
role for this growth factor only in the early phases in
20   bone progenitor cell development.

One of the difficulties in dissecting cell lineages
is the need to define precisely developmental stages.
The inventors combined immune-isolation, flow cytometric
25   multiparameter analysis, and functional assays (colony-
formation and growth factor responsiveness) to
characterize human marrow-derived bone precursor cells.
Thus, the proliferative component of this lineage
consists of two types of osteoprogenitor cells (the colony-
30   and cluster-forming cells) as well as the
preosteoblasts, each distinguished by their cell-size,
growth factor responsiveness, and proliferative
potential. The remaining isolated bone cells are the
osteoblast cells that express increased amounts of bone
35   proteins in response to TGF-β1, have a low proliferative
potential (if any) and elaborate and mineralize a
collagen and non-collagenous bone protein-containing

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extracellular matrix (Long *et al.*, 1990). These studies
show that osteoprogenitor cells are antecedent to bone-
forming cells in the following differentiation cascade:
Colony forming cell → *Cluster forming cell* →
5 *Preosteoblast* → *Osteoblast.*

Bone precursor cells co-exist among a population of
hematopoietic progenitor cells which can be separated
from their differentiated progeny by physical methods
10 (i.e., equilibrium density separation, plastic-
adherence). Bone marrow-derived bone precursor cells
were not hematogenous in origin, as these cells did not
express the pan-hematopoietic cell surface antigen CD34,
and failed to respond to hematopoietic growth factors
15 (Long *et al.*, 1990). Moreover, unlike the murine system,
human bone marrow-initiated bone cell cultures can not be
derived from bone marrow stromal cells (Long *et al.*,
1990).

20 Finally, in later phases of differentiation (i.e.,
in serum containing, long-term cultures), bone marrow
derived osteoblasts produce an extracellular matrix which
contains Type I collagen, osteonectin, osteocalcin, and
is positive in the Von Kossa reaction; these bone cells
25 also were capable of depositing calcium ($^{45}Ca^{++}$) into the
extracellular matrix (Long *et al.*, 1990). These
observations and the present data show that a separate
lineage of osteopoietic cells exists within human bone
marrow. The data also show that, at some stage during
30 their ontogeny, bone precursor cells such as the
preosteoblast and, in particular, the osteoprogenitor
cell are not intimately associated with the endosteal
surface of bone. Rather, these cells may migrate to the
endosteum upon acquisition of certain developmental
35 characteristics, such as the expression of bone protein
(or other extracellular matrix) receptors. Osteogenic

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precursors thus exist as a reservoir of bone forming
cells within the bone marrow.

Example 2:   Alterations in osteoprogenitor cell
                 responsiveness to osteogenic cytokines
                 during the aging process

The process of aging is associated with a
progressive diminution of bone forming capacity,
especially in trabecular bone (Roholl et al., 1994 and
Nimni et al., 1993. This process is associated with a
number of alterations in bone proteins, osteoid
formation, calcium loss etc. leading to osteopenia.
Central to all of this is the osteoblast. Reduction in
osteoblast numbers of necessity leads to the loss of bone
forming capacity. One potential mechanism for such a
reduction in osteoblast numbers is a decreased
responsiveness of the osteoprogenitor cells to mitogenic
activation. As a result, a differentiation blockade may
exist somewhere between the osteoprogenitor cell and the
osteoblast. Such a blockade has been demonstrated in a
rat model system, in which morphometric analysis
indicated both a reduction in trabecular bone volume and
a (morphological) ten-fold decrease in osteoblast numbers
with age (Roholl et al., 1994).

Similarly, a morphological study in humans also
indicates an age-related decrease in osteoblast numbers
(Nimni et al., 1993). Other studies have indicated that
osteoblast cells show a reduced responsiveness to
osteogenic growth factors in both humans (Pfeilschifter
et al., 1993), and mice (Wong et al., 1992). Again,
information on the cytokine responsiveness of human
osteoprogenitor cells or preosteoblasts is lacking. The
reduction in human osteoblast numbers is likely due to
age-related reductions in growth factor responsiveness of
the osteoprogenitor cells.

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With the inventors' ability to quantify two types of osteoprogenitor cells, preosteoblasts, and osteoblasts, the inventors can quantitatively define the level at which such a block occurs for human cells. If a blockade

5   exists between the osteoprogenitor cell and preosteoblast then numbers of the former will increase in frequency with an attendant decrease in a preosteoblast numbers. A similar alteration in cell ratios is detectable if the blockade is between the preosteoblast and osteoblast.

10

Age-related changes in cell proliferation are evaluated by stimulating bone precursor cells by various osteogenic cytokines. Certain growth factors (or cytokines), in particular TGF-1 and bFGF, differentially

15 stimulate the early phases of osteoprogenitor cell proliferation. The role of differing cytokines in the proliferation of bone precursor cells in two age groups, 18-25 and $\geq$50 are of interest. Osteoprogenitor, preosteoblastic, osteoblastic cultures from both age

20 groups with varying concentrations of osteogenic growth factors are cultivated. Given their effect on proliferation, growth factors TGF-β, BMP2, bFGF and 1,25 vitamin D3 are utilized. Other growth factors such as PTH and other BMP family members are also the subject of

25 investigation. Comparison of cell growth for both age groups utilizes all the quantitative methods described above. In addition to concentration dependency, age-related differences in temporal responsiveness to growth factors are examined.

30

The differential effects for various cytokines at the early and late phases of bone precursor cell proliferation (e.g., TGF-β, and 25-OH vitamin D3, respectively) are investigated. A synergistic

35 interaction may occur if bone precursor cells are cultured simultaneously with these two classes of growth factors. The inventors will thus evaluate a number of

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relevant combinations of growth factors e.g., TGF- + BMP;
TGF- + D3; bFGF + BMP and bFGF + D3.

5   These studies on cytokine control of osteoprogenitor
cell proliferation allow precise determination of any
age-related changes in cytokine responsiveness. In
addition, these studies generate important information
concerning combinatorial cytokine control of bone cell
development. The inventors' ability to examine the
10   osteoprogenitor cells, preosteoblasts, and osteoblasts
pinpoints the exact developmental level at which
alterations such as a differentiation blockade occur.
For example, age-based alterations may show that, while
osteoprogenitor cell proliferation is equivalent, a
15   reduced responsiveness of preosteoblasts exists which
indicates a defect at this level of development. Thus,
each of the three assay types accesses alternative levels
of differentiation for defects. Each of the components
of the osteogenic microenvironment, purified target
20   cells, purified (and/or) recombinant cytokines/growth
factors, and purified extracellular matrix molecules) are
available and their interactions are explored in a
systematic, controlled fashion. Thus, both positive and
negative results can be easily detected at all steps of
25   bone precursor cell proliferation, in both age groups.
These studies are limited in that they do not evaluate
other types of control such as ECM proteins or failure of
osteoblasts to differentiate. These are addressed below
in Example 3.
30

**Example 3:   Cellular interactions during
osteoprogenitor cell proliferation and
differentiation, and how they are
modulated during aging**

35

As is true with other tissue types, bone cell
development occurs in a specific microenvironment in

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which developing bone cells interact with each other, the
extracellular matrix, and with matrix:cytokine complexes.

5   An important but poorly understood component of the
osteogenic microenvironment is the extracellular matrix.
As mentioned, bone extracellular matrix contains both
collagenous and non-collagenous proteins. When bone
precursor cells are cultured on certain non-collagenous
proteins, they show an increase in proliferation, and in
10   bone cell antigen expression. Moreover, the inventors
have shown, using the hematopoietic system as a model,
that subpopulations of primitive progenitor cells require
both a mitogenic cytokine and a specific extracellular
matrix (ECM) molecule in order to proliferate (Long et
15   al., 1992). Indeed, without this obligate
matrix:cytokine ("matricrine") signal, the most primitive
of blood precursor cells fail to develop in vitro (Long
et al., 1992). Although poorly understood, it is likely
that a similar requirement exists for human bone
20   precursor cells. Complete evaluation of osteogenic
changes occurring with age requires an understanding of
the role of ECM molecules in bone precursor cell
proliferation.

25   As mentioned, developing tissue cells interact with
a wide variety of regulators during their ontogeny. Each
of these interactions is mediated by defined, specific
receptor-ligand interactions necessary to both stimulate
the cell proliferation and/or motility. Also, both
30   chemical and/or extracellular matrix gradients exist
which signal the cell to move into a defined
microenvironment (e.g., into the region of a bone
fracture). As well, high concentrations of the
attractant, or other signals, next serve to "localize"
35   the cell, thus stopping its non-random walk. Signals
which stop and/or regionalize cells in appropriate
microenvironments are poorly understood.

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The inventors also have shown, in the hematopoietic
system, that complexes of cytokines and extracellular
matrix molecules serve to localize progenitor cells (Long
et al., 1992). Similar mobility (chemotactic) or
5   localization signals exist for bone precursor cells, and
mediate their movement into an osteogenic region (such as
a fracture). Importantly, the inventors have devised a
means to examine each of these phenomena. Quantitative
assays for cell adhesion provides the minimal
10   requirements for cell localization. Quantitative assays
for assessing both chemotaxis and chemokinesis are also
utilized.

Cell:Cell Interactions Required For Bone Precursor Cell
15   *Proliferation.* The effects of aging on human bone
precursor cells is evaluated by examining cell:cell and
cell:ECM interactions at the various stages of
development. The role of aging on the expression of
known cell adhesion molecules is evaluated, particularly
20   focusing on the β1 and β3 integrins. The Integrin Gene
Superfamily members serve as receptors for both other
cells and extracellular matrix proteins (Giancotti et
al., 1990). Cell attachment to integrins is rapid
(within minutes) and occurs as a result of increased
25   avidity rather than increased expression (Lawrence et
al., 1991). The ligand for most, but not all, integrins
is the tripeptide sequence arginine-glycine-asparagine
(RGD) (Ruoslahti et al., 1987).

30   Structurally, integrins are comprised of two
membrane-spanning alpha and beta chains. The
alpha-subunits contain three - four tandem repeats of a
divalent ion binding motif and require magnesium or
calcium to function. The alpha chains are (for the most
35   part) distinct and bind with common or related β-subunits
to yield functional receptors (Giancotti et al., 1990).
The β chains seem to have functional significance and

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integrins can be subclassified based on the presence of a
specific beta chain. Thus, family members containing $\beta 1$
and $\beta 3$ chains predominantly subserve cell:extracellular
matrix interactions whereas molecules containing the
5 $\beta 2$-subunits primarily function in leukocyte:leukocyte
interactions.

The studies thus focus on the role of $\beta 1$ and $\beta 3$
integrins in mediating the formation of human bone
10 precursor cell colonies and preosteoblast proliferation.
Two alternative approaches are used. First, antibodies
to specific integrins (the VLA proteins 1-6, fibronectin
receptor, type I collagen receptor and vitronectin
receptor, as well as two non-integrin adhesion molecules
15 ICAM 1 and 2) are used as probes for integrin expression
on human bone precursor cells by flow cytometry.

Once the relevant molecules are identified, their
role in both proliferation and differentiation formation
20 will be evaluated in the inventors' bone cell assays. In
these studies, human bone precursor cells assays (as
above) are established and treated with antibodies (as
well as isotype specific controls) to determine the role
of these receptors in colony formation (i.e., cell:cell
25 interactions at the osteoprogenitor cell to preosteoblast
level), and in preosteoblast cultures (to examine their
role in the proliferation and in the preosteoblast to
osteoblast step). Alternatively, these cultures are
treated with peptides containing the RGD sequence to
30 inhibit integrin-mediated function. Control cultures are
treated with equivalent sized peptides lacking the RGD
motif.

Bone Precursor Cell:Extracellular Matrix Interactions.
35 The inventors have demonstrated the importance of three
bone ECM proteins in human bone cell growth: osteonectin,
osteocalcin, and type I collagen (Long *et al.*, 1990 and

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Long *et al.*, 1994). These and four additional matrix
proteins are evaluated for changes occurring during
aging: bone sialoprotein, osteopontin, fibronectin, and
thrombospondin (Nomur *et al.*, 1988 and Oldberg *et al.*,
5   1986). Bone sialoprotein and osteopontin are likely to
be involved in bone formation but their role in
proliferation is unknown. The latter two proteins
(thrombospondin and fibronectin) have been included due
to their presence in bone extracellular matrix, and their
10   demonstrated importance as cytoadhesion molecules in
developing tissues (Weiss *et al.*, 1980 and Clezardin *et
al.*, 1989).

       In order to evaluate the actions of these ECM
15   molecules on cell proliferation, soluble, purified bone
ECM proteins are added to cytokine-driven bone precursor
cell cultures. Varying amounts of exogenous ECM
molecules are added in the presence of a single growth
factor (e.g., 25 pM TGF-, or the optimal factor
20   concentration defined as detailed above), to allow
precise evaluation of the developmental effects of each
extracellular matrix molecule. The combined effects of
relevant cytokine and matrix molecules on the expansion
of human bone precursor cells in vitro are determined.
25

Bone Precursor Cell Cytoadhesion and Tissue Localization
A cytoadhesion assay which employs "caged" fluorochromes
to label isolated progenitor cells for subsequent
adhesion studies has been developed. In this assay,
30   acetylmethylester derivatives of FITC are used to
viably-label the cells. Upon internalization,
intracellular esterases cleave the AM-ester derivative
rendering the released fluorochrome relatively
impermeable. Importantly, the fluorescence signal is
35   linear with respect to cell number, and as few as several
hundred cells can be detected. The cytoadhesion assay
consists of the adhesion of caged-fluorochrome labeled

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cells to purified and/or recombinant proteins which are
immobilized onto tissue culture plastic, as described
previously (Long and Dixit, 1990 and Long et al., 1992),
the removal of non-adherent cells, and quantitation in a
5   fluorescent plate reader. The resultant sensitivity of
this assay is approximately 100 times greater than other
cytoadhesion assays reported (Long and Dixit, 1990; Long
et al., 1992; Campbell et al., 1990).

10   The inventors utilized this assay in preliminary
studies of purified human bone precursor cells (from
young individuals) to evaluate attachment to
extracellular matrix molecules. These data indicate that
bone precursor cells express differential attachment
15   capacities to both immobilized bone ECM molecules and
immobilized cytokines. These observations are similar to
previous work of the inventors' laboratory in which
hematopoietic progenitor cells were demonstrated to bind
to both growth factors and ECM molecules (Long and Dixit,
20   1990; Long et al., 1992; and Campbell et al., 1990).

Thus, as divergent cellular phenotypes, bone and
hematopoietic cells both demonstrate dual requirements
for matrix and cytokine molecules in the localization
25   (adhesion) process. Notably, the binding of progenitor
cells to immobilized, solitary cytokines further
demonstrates that the presence of growth factors (which
are often themselves immobilized within the extracellular
matrix (Long, 1992), is as least partially responsible
30   for the lineage-specific localization of cells.
Nonetheless, the presence of specific ECM molecules
undoubtedly strengthens this localization process.

To perform these studies, cultures of proliferating
35   bone precursor cells as well as osteoblast cells are
established under optimal cytokine conditions (as above).
The capacity of bone precursor cells to interact (adhere)

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with bone cell regulatory cytokines (bFGF, TGF-1, and
BMP-2 and extracellular molecules (osteonectin,
osteocalcin, bone sialoprotein, osteopontin, fibronectin
and thrombospondin) are evaluated. These proteins are
5   studied individually, and in combination, in order to
determine their relative, or synergistic contribution to
bone cell adhesion. These studies both corroborate and
extend studies in which ECM cytokine molecules are
exogenously added to the cultures to assess proliferative
10   effects.

Bone Precursor Cell Chemotaxis. In a likewise fashion,
the effects of aging on the motility machinery of
developing bone precursor cells are evaluated. In these
15   studies, various bone related growth factors are
evaluated for their capacity to direct non-random
movement (cytokinesis) and non-random migration
(chemotaxis). As mentioned, early bone precursor cells
possess the ability to actively migrate into the area of
20   bone injury, there differentiating into bone-forming
cells. However, no information exists on the factors or
events which signal this important migratory process or
how it may be altered during aging. A fluorescence-based
assay which evaluates both chemokinesis and chemotaxis is
25   available (Deforge *et al.*, 1992).

In order to evaluate the effects of age on bone
precursor cell migratory capabilities, a panel of known
leukocyte chemotactic factors, osteogenic factors and ECM
30   proteins in direct comparison of cells from the two age
groups are utilized. Bone precursor cells from
individuals of both age groups are evaluated for
responsiveness to both known chemotactic factors
(chemokines; i.e., interleukin-8, GM-CSF, M-CSF) and for
35   the putative role of osteogenic growth factors in
stimulating either chemokinesis or chemotaxis. In
particular, bFGF and TGF-1, both powerful regulators of

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bone progenitor cell proliferation are evaluated as well
as BMP2, PTH, and 1,25-OH vitamin D3. The chemokines are
members of a chemotactic cytokine supergene family
(Oppenheim et al., 1991).

5

The "chemokine-" cytokines are comprised of
molecules with their first two cysteines interrupted by
an amino acid (C-X-C), and are represented by such
molecules as interleukin-8 (IL-8) and platelet factor 4
10   (PF4). MCP-1 and RANTES are representative of the
"chemokine-" subfamily, and are characterized by an
uninterrupted C-C arrangement. The use of IL-8 and MCP-1
allows employment of chemotactic factors which are known
to induce migration of a broad spectrum of cells
15   (Oppenheim et al., 1991).

The ECM proteins evaluated are described above.
These studies determine (Urist et al., 1983) the
appropriate cytokine and/or cytokine combination for bone
20   precursor cell growth (Fishman et al., 1962), the role of
cell:cell interactions, and (Hattersley et al., 1989) the
relevant ECM molecules necessary for human bone precursor
cell proliferation. The studies on integrin expression
detail the mechanism of how human bone precursor cells
25   interact with each other, the ECM, and how these
interactions may be modified during aging. Studies of
ECM molecules determine which are the relevant components
involved in proliferation and/or migration, and whether
responsiveness to any of these is lost with age.

30

Additionally, it is likely that excess
concentrations of a given cytokine(s) or ECM protein will
overcome an age-related deficit, thus demonstrating that
the age defect is exogenous to the cell. These studies
35   are designed such that a complex osteogenic
microenvironment is reduced to a stepwise evaluation of
each of its relevant components. These studies define

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both the minimum essential conditions for the
proliferation of human bone precursor cells and
demonstrate age-related changes.

5   **Example 4:**   **Age-Related Alterations in Bone Protein**
                        Expression by Purified Populations of
                        Human Bone Precursor Cells

        The present example concerns the further
10 immunological purification and characterization of bone-
    antigen positive human trabecular bone precursor cells,
    and the demonstration that distinct, age-related changes
    in the cellular expression of osteonectin and osteocalcin
    occurs in these cell types.
15

    MATERIALS AND METHODS
    Bone Marrow Cell Preparation and Culture.   Human bone
    marrow aspirates were obtained from normal volunteers
    following informed consent.   It should be noted that
20 samples from young individuals (≤ 18 years) came from the
    discarded portions of bone marrow obtained on the
    Pediatrics Hematology/Oncology Service for the purposes
    of allogenic bone marrow transplantation, for which
    informed consent was granted by the parent(s).   Bone
25 Marrow samples from individuals ≥ 18 years of age were
    obtained following informed consent by routine bone
    marrow aspiration.

        As an alternative, marrow from older individuals was
30 also obtained from rib fragments discarded in the course
    of thoracic surgery (to date, the inventors have detected
    no site-dependent differences in flow-cytometry
    parameters of human bone precursor cells).

35   Bone marrow non-adherent low density (NALD) cells
    were prepared by adherent cell depletion, and density
    separation techniques as described by Long *et al.* (1988)

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and in Example 1. NALD cells are then subjected to
immune-adherence isolation, or immuno-magnetic
purification.

5   *Antibodies, Immune-Adherence, and Induction of
        Differentiation.* The expression of bone protein antigens
        was determined by fluorescence activated flow cytometry
        using monoclonal antibodies to human osteonectin,
        alkaline phosphatase, or osteocalcin, as described in
10   Example 1.

        Immune-adherence isolation of human trabecular bone
        precursor cells and their induction into osteoblast
        differentiation was performed as described in Example 1.
15

        The induction of osteoblast differentiation was
        performed as described in Example 1 using 25 pM TGF-β.

        Immunomagnetic Purification of Human Bone Precursor
20   *Cells.* Purification of human bone precursor cells was
        performed using immunomagnetic separation. This was
        carried out *via* Magnetic Activated Cell Sorting (MACS;
        Miltenyi Biotec, Sunnyvale, CA) as per manufacturer's
        instructions, but modified as noted below.
25

        For MACS separation, the NALD cells are washed once,
        and simultaneously labeled with FITC-conjugated
        monoclonal anti-osteocalcin and biotinylated anti-
        osteonectin (both as above), both at 10 μg/mL, in Tris-
30   Buffered Saline containing 1% BSA, pH 7.6 The antibody-
        labeled cells are then washed twice, and labeled with
        goat anti-mouse IgG (heavy and light chain specific;
        Miltenyi), conjugated to superparamagnetic nanobeads.
        The antibody is incubated at 180 μL per 10^8 NALD cells
35   per 1.0 mL for 30 min, at 4°C.

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The anti-IgG labeling is performed in column-loading
buffer (0.5% BSA, 0.1% glucose in PBS), modified to
contain 0.5 μg/mL aprotinin, 0.5 μg/mL leupeptin, and
5 μg/mL soybean trypsin inhibitor, and 500 U/mL of DNase
5   (Sigma). The protease inhibitors were added following
preliminary studies which demonstrated a progressive loss
of antigens with increasing separation time; the DNase
was added to eliminate cell clumping (Long et al., 1988).

10   From 100-300 × 10^6 cells are loaded onto a Miltenyi
magnetic column equipped with a 26g flow-restrictor
(Miltenyi) at 1 × 10^8 cells/mL in 1.0 mL increments,
running 0.5 mLs of loading buffer after each increment.
Antigenically negative cells are eluted with 1.0 mL of
15   elution buffer (loading buffer with DNase and protease
inhibitors increased by a factor of 4). Following
elution antigen-positive cells are recovered by removing
the magnet, and eluting with 1.0 mL of elution buffer.
These antigen-positive cells are then re-isolated on a
20   second magnetic column using the same procedure.

Flow Cytometric Analysis. Flow cytometric analysis was
performed as described in Example 1 using a Becton-
Dickinson (San Jose, CA). FACSCAN system and data
25   analyzed with the BD LySys software program. Controls
consisted of autofluorescence as well as non-specific
fluorescence detected with isotype-specific murine
monoclonal antibody to keyhole lymphet hemocyanin (KLH)
obtained from Becton Dickinson.
30

For two-antigen (two-color) analysis, the
magnetically isolated cells are then incubated for 15 min
at 4°C with strep-avidin conjugated to PerCP (Becton
Dickinson) in order to visualize the osteonectin
35   antigenic determinant. Immunomagnetically purified cells
were analyzed for fluorescence intensity versus size

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(forward-angle scatter), as well as intracellular
complexity (side-scatter).

5   In order to map the cellular characteristics of
preosteoblast and osteoblast cells in immune-isolated
populations the two-color fluorescence profiles were
back-gated (Given, 1992) to determine their size and
side-scatter profiles.

10   *RESULTS*
Osteoblastic Differentiation of Preosteoblasts. The
inventors previously demonstrated that immune adhesion
mediated by antibodies to human bone matrix proteins
results in the isolation of population of human bone
15   marrow which are responsive to osteogenic cytokines, but
lack responsiveness to hematopoietic growth factors
(Example 1).

        In order to further characterize bone precursor
20   cells, the inventors isolated human bone precursor cells
by immune adherence and subjected the resultant cell
population to multiparameter flow cytometry to
characterize the cellular differences between isolated
pre-osteoblasts and osteoblasts.
25
        Confirming and extending the previous data, immune-
adhesion yielded a population of bone antigen-positive
cells, that predominantly are the size of a lymphocyte
which, when stimulated with TGF-β, show a marked increase
30   in antigen density and cell size (FIG. 5A, FIG. 5B and
FIG. 5C). Notably, there are two size (as indicated by
the parameter "Forward angle scatter" in FIG. 5)
populations among immune adherent cells, and TGF-β-
induced differentiation results in a shift between the
35   small and large cell compartment (FIG. 5A, upper left and
right panels).

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In order to further examine the developmental
characteristics of these cells, isolated uninduced and
induced cells were electronically divided into small and
large cell compartments and "back-gated" to determine the
5   side-scatter characteristics of each (FIG. 5A, lower left
and right sub-panels).

These data show that the larger osteoblast cells
(i.e., those falling within the outlined square in
10   figures) also have the highest degree of intracellular
organization (side-scatter characteristics). This was
seen in both uninduced cells (in which osteoblast-sized
cells represent approximately 5% of the isolate) and
induced cells (FIG. 5, left and right sub-panels,
15   respectively). Thus, the TGF-β-induced differentiation
of human bone precursor cells results in an increase in
antigenic content, cell size, and an increase in
intracellular complexity. This is true for both
osteocalcin (BGP)-positive cells, as demonstrated in
20   FIG. 5A, FIG. 5B and FIG. 5C, and alkaline-phosphatase-
positive cells.

Isolated cells also contain a population of cells
(circled in FIG. 5A) in which antigen density is little
25   different from non-specific antibody controls. Back-
gating of this population consistently shows these cells
as having a low degree of side-scatter. These
characteristics (i.e., low side-scatter and small size)
are consistent with a residual population of lymphoid-
30   like which do not bear detectable bone antigens.

*Immunomagnetic Purification of Human Bone Precursor
Cells.* Immune-adherent isolation of human bone precursor
cells is based on the co-immobilization of monoclonal
35   antibodies to both osteonectin and osteocalcin
(Example 1). This procedure results in a purified
population of bone precursor cells which express

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osteocalcin, osteonectin, and alkaline phosphatase.
However, the procedure does not allow for distinguishing
between individual subpopulations of cells expressing
these antigens, or a single population of cells which co-
5   express osteonectin, alkaline phosphatase, and
osteocalcin on the same cell.

In order to examine this, the inventors undertook
further purification of these cells utilizing magnetic-
10   activated cell sorting. The isolation of these cells
thus uses physical separation (equilibrium density
centrifugation), plastic-adhesion (to remove bone marrow
stromal cells), and immunomagnetic separation.

15   Interestingly, physico-chemical separation (density)
results in a moderate enrichment of these cells to a
level of 6-7% purity (FIG. 6A; Table 1). In contrast,
immuno-magnetic separation based on the osteocalcin and
osteonectin antibodies antigens yields a 95% pure
20   population of cells (FIG. 6B) which represents an
approximate 4,800-fold purification over unfractionated
bone marrow (Table 1).

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TABLE 1

Purification of Human Bone Precursor Cells by Combined
Physico-Chemical and Immunological Procedures.

	Procedure	HBPC Frequency	HBPC fold - Purification
5	Unfractionated BM[a]	0.0002	N.A.
	Post-EDC and plastic- adherence (NALD)	0.06	300
10	Post-Immune-[b] adherence separation	0.60	3000
	Post-Immuno- magnetic separation	0.95	4750

15   Frequency is number of osteocalcin-positive as
     determined by flow cytometry.

[a] Unfractionated bone marrow is frequency of
     osteocalcin-positive cells in un-separated bone
     marrow in which RBCs are removed by velocity
20   sedimentation and the resultant WBC subjected to
     flow cytometry and back-gating as in text.

[b] Immune adherence and immunomagnetic separation
     purification are *not* sequential steps. Rather,
25   these two rows show the relative fold-purification
     of each technique.

     BM = bone marrow, EDC = equilibrium density
     centrifugation, HBPC = human bone precursor cells,
30   NA = not applicable, NALD = Non-adherent low
     density. A typical separation procedure is
     presented.

35   Immunomagnetically separated cells were subjected to
     two-color fluorescence-activated cytometry to examine the
     expression of osteonectin and osteocalcin. These data

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show that the isolated cells co-express both proteins
(FIG. 8B). Moreover, antigen-density contour plots
demonstrate that these antigens are co-expressed in a
single population of cells, in that no distinct sub-
5   populations of single-antigen positive cells are
detected.

There remains, however, a small population of
antigen-dull cells (quadrant 3 in FIG. 6B). However,
10   unlike the antigenically null-population seen in cells
separated by immune-adherence, the magnetically separated
antigen-low or dull cells have the same side-scatter
characteristics as the double-positive cell population
(compare FIG. 5B, circled population right and left).
15   Given that these cells are recovered following two passes
through the magnetic isolation column, it is unlikely
that these are contaminating lymphoid cells (as are seen
in immune adherence-based separation). Rather, they
represent cells with a sufficient (albeit low) antigen
20   density (albeit a low density) to retain them on the
column in the presence of a magnetic field.

*Increasing Donor Age is Associated with Alterations in
Bone Protein Expression of Human Bone Precursor Cells.* A
25   series of flow cytometric investigations on
immunomagnetically-separated bone precursor cells
demonstrate that age-related changes in bone protein
expression occur with increasing age. These studies were
performed on a total of 41 individuals of three age
30   groups: ≤ 25 years old (mean age 16.4 ± 7 (S.D.) years,
range 1.5-24 years, n=15), 50 years old; (mean age 36.6
± 5 years old, range 26-45 years, n=9) and individuals
≥ 50 years old (mean age 70.1 ± 12 years, range 53-89
years, n=17).

35

Human bone precursor cells were isolated and
purified cells from individuals in the given age groups

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and subjected to multi-parameter flow cytometric
analysis. Confirming the above observations,
antigenically-purified human bone precursor cells from
these three age populations co-express both osteonectin
5   and osteocalcin.

Interestingly, osteonectin and osteocalcin antigenic
expression by human preosteoblast cells increases with
increasing age. In order to visualize these shifts in
10   antigen density, cytometric profiles from two
representative individuals (one old-aged 60, one young-
aged 9) were overlaid (FIG. 7A and FIG. 7B). These
profiles were defined as representative because their
mean-fluorescence and peak-fluorescence values were
15   identical to the averages determined for their respective
age-cohorts, and their coefficiencies of variation were
similar.

FIG. 7A and FIG. 7B clearly illustrates that the
20   human bone precursor cells in older individuals (i.e., $\geq$
50 years of age) express higher amounts of these two bone
proteins than do younger individuals (i.e., $\leq$ 25 years
old). Profiles from middle aged individuals were
intermediate to the other two age groups.
25

In order to determine whether these alterations were
statistically significant for the whole population, the
mean specific- and peak-fluorescence were determined for
each individual in each age group. A significant
30   ($p \leq 0.05$) age-related increase was noted in both the
mean specific fluorescence for osteocalcin and
osteonectin (FIG. 8), as well as in the peak fluorescence
of each antigen.

35   Osteocalcin shows a moderate but significant change
in mean fluorescence, increasing by 21 arbitrary log
units. In contrast, osteonectin expression increases to

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a greater degree in the population of older individuals (an increase from 59 to 89 arbitrary log units; FIG. 10).

5   The inventors further analyzed this unexpected increase in bone protein levels by examining the relationship between age and antigenic expression (FIG. 9). Note that, in this data, the numbers of individuals of middle age are lower than the other two age-cohorts. Nonetheless, it appears that the majority
10   of the increase in human bone precursor cell osteonectin and osteocalcin levels occurs between the ages of 15-16 and 35-40.

         Of the seventeen individuals greater than 60 years
15   of age evaluated, four (three women, age 64, 88; and 89 years old; one male age 59) did not show the human bone precursor cell immunophenotypic characteristics described above (FIG. 5C). Rather, the majority (80-90%) of the presumed preosteoblasts isolated from these individuals
20   are similar in antigenic content to the antigen-dull population of cells described in FIG. 5B (left hand panel, circled population).

         These individuals thus lack the predominant
25   osteocalcin-positive/osteonectin-positive (OC^{++}/ON^{++}) bright population observed in all other individuals (n=37), including their age-related cohort (both male and female). Given the immunomagnetic retention of these cells on the column, and that the forward-angle and side-
30   scatter characteristics of these cells are identical to that of the antigen-dull cell subpopulation, the inventors conclude that these individuals possess a population of preosteoblasts which differ greatly in their antigenic expression.
35

         Consistent with this, the mean and peak fluorescence of both osteonectin and osteocalcin are significantly

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reduced, even when compared to the youngest age-cohort
(Table 2). These data suggest that a separate
subpopulation of individuals exists with distinctly
different (lower) human bone precursor cell antigenic
5   content.

The physiological and clinical significance of such
alterations may be that the immunophenotype of these two
populations of elderly individuals reflects the status of
10   their bone cell function. By their sixty-seventh decade
of life, most individuals (male and female) show varying
degrees of osteoporosis. Thus, the identification of
alterations in bone protein expression undoubtedly
demonstrates the basis for known elevations in these
15   proteins (osteocalcin (BGP) and osteonectin) in the
plasma of elderly individuals. The identification of a
subpopulation of elderly individuals might thus
demonstrate a group of individuals with more severe
disease.

20

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TABLE 2

Comparison of Bone Protein Expression
Among Elderly Individuals

	Osteocalcin (BGP)			Osteonectin	
Age (yrs.)	Peak Fl.	Mean FL.	Peak FL.	Mean Fl.
59	70	63	17	25
64	54	52	33	40
88	59	59	43	36
89	63	65	36	31
Mean:	75 ± 8	62 ± 3*	60 ± 3*	30 ± 4*	33 ± 3*
Cohort Mean:	69 ± 3	122 ± 7	123 ± 6	91 ± 8	89 ± 7

* = $p \leq 0.001$ (Student's t-test, two tailed)
compared to the age-related cohort,
Fl = fluorescence. Summary values are mean ± S.E.M.

Example 5:   Preparation of Bone Precursor Cells from Peripheral Blood

The present example concerns the further preparation of bone precursor cells from peripheral blood.

The inventors found that animal bone precursor cells circulate, thus making it possible to recover and purify these cells in animal peripheral blood.

Human bone precursor cells were isolated from human peripheral blood by venipuncture. Blood was drawn into preservative-free heparin, to prevent coagulation, and processed for human bone precursor cells using the same protocol as described above for bone marrow isolation via immunomagnetic separation. Briefly, this entails equilibrium density centrifugation (ficoll), and plastic-adherence to generate NALD cells, their labeling with

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monoclonal antibodies to osteocalcin (BGP) and
osteonectin, and processing for immunomagnetic separation
and purification. The resultant cells were analyzed by
multi-parameter flow cytometry as described above.

5

The human bone precursor cells isolated from
peripheral blood were found to have similar flow
cytometry characteristics to those of bone marrow.

10   **Example 6:**   **The effect of aging on the ability of bone
                        precursor cells to differentiate into
                        osteoblast cells and the capacity of such
                        osteoblasts to elaborate an osteoid
                        extracellular matrix**

15

The complete evaluation of bone cell development
requires the study of both bone precursor cells
(osteoprogenitor cells and preosteoblasts) as well as
their differentiated progeny, the osteoblasts. It is the
20   osteoblasts that are responsible for the elaboration and
mineralization of bone matrix, and the subsequent
formation of bone. As mentioned previously, published
morphological and morphometric reports suggest that a
differentiation blockade may occur during the aging
25   process resulting in reduced osteoblast numbers (Roholl
et al., 1994 and Nimni et al., 1993). However, other
evidence exists suggesting that the osteoblasts in older
individuals have diminished function in that they show
decreased responsiveness to mitogenic stimuli
30   (Pfeilschifter et al., 1993 and Wong et al., 1992), and
produce decreased amounts of bone matrix proteins, (Liang
et al., 1992) and osteoid (Nimni et al., 1993).

It is important to evaluate the capacity of
35   preosteoblasts to differentiate into osteoblasts, and the
capacity of those osteoblasts to function normally.
Importantly, such studies cannot be performed on human

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trabecular bone outgrowth cultures for a variety of
reasons (limiting cell numbers, lack of purified cells,
heterogeneity of cell types present, etc.), and because
the principle age-related defect may be a failure earlier
5   in the osteoblast differentiation pathway. That is, cell
development may not progress from osteoprogenitor cell to
preosteoblast, or from preosteoblast to osteoblast.
Therefore, it is best to examine this problem in a system
in which the full spectrum of human bone cell
10   proliferation/differentiation can be evaluated.

Regulation of Osteoblast Cell Differentiation. As
described above, the inventors have developed a system in
which isolated and purified bone precursor cells
15   proliferate and differentiate into osteoblasts. In
particular, the inventors have demonstrated that the
process of switching serum-free cultures to TGF-β driven,
serum-containing cultures results in a differentiation of
preosteoblasts into osteoblast cells (Long *et al.*, 1990).
20

Importantly, the osteoblasts generate an osteoid
extracellular matrix in which non-collagenous bone
proteins are deposited and the cells calcify the
extracellular matrix (Long *et al.*, 1990). The effect of
25   aging on both the differentiation of preosteoblasts into
osteoblasts, and the subsequent alterations in
differentiated cell (osteoblast) function is being
investigated. As such, the cell culture protocol
described herein is modified to induce osteoblast cell
30   differentiation.

In these studies, bone precursor cell cultures are
run in two phases. The first phase (7-10 days) is a
proliferation step utilizing serum-free cultures
35   containing either TGF-β or, if better, a growth
factor/matrix combination for generation of maximum
numbers of bone precursor cells. During this phase of

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the cultures, only cellularity and antigen expression is
monitored as an index of culture conditions. At optimal
cell density, these cultures are switched to
serum-containing, TGF-β-containing conditions.

5

Subsequent studies (over an additional 7-10 days)
evaluates osteoblast differentiation patterns and
functional capacities. As described herein, the inventors
have defined a multi-parameter flow cytometric analysis
10   of osteoblast cell differentiation markers; these cells
increase in cell size, cell complexity, and the
expression of multiple markers of bone cell
differentiation. The capacity of pre-osteoblasts to
differentiate into osteoblast cells using this
15   multi-parameter flow cytometric analysis is determined.

In particular, the expression of the bone proteins,
osteocalcin and osteonectin, plus two additional
biochemical markers of bone differentiation, alkaline
20   phosphatase and type I collagen is determined. As
mentioned, alkaline phosphatase are monitored by both
flow cytometry, as well as by alkaline phosphatase (EC
3.1.3.1) cytochemistry (Reddi, 1981). This latter
procedure distinguishes bone alkaline phosphatase from
25   that of liver based on heat and urea sensitivity. As a
corroborative study, the production and deposition of
bone extracellular matrix molecules by osteoblast cells
from both age groups is determined by metabolic labeling
and immunoprecipitation using ^{35}S-methionine
30   immunoprecipitation.

Extracellular Matrix Calcification. Studies on the
capacity of osteoblast cells to mineralize their
surrounding (bone) ECM employ two alternative approaches:
35   metabolic labeling with ^{45}Ca^{++}, and histochemical
analysis utilizing the von Kossa staining procedure. For
the calcium labeling studies, two aspects of bone cell

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calcium metabolism are evaluated: their ability to take up calcium and their ability to deposit calcium into the extracellular matrix.

5   As previously demonstrated, osteoblast cells are removed from culture and equilibrium-labeled (one hour) with $^{45}Ca^{++}$ (Long *et al.*, 1990 and Long *et al.*, 1993). Briefly, osteoblasts cultured for 3, 5, 7, 14 and 21 days post serum-, or serum-free growth factor stimulation are
10   removed and washed 3 times with calcium-free PBS, and metabolically labeled with 50 Ci $^{45}Ca^{++}$ for 60 min at 37°C. Following $^{45}Ca^{++}$ calcium-equilibration, labeled cells are washed free of unincorporated calcium, resuspended in serum-free tissue culture medium and
15   re-established in the original culture dishes. An aliquot of Ca^{++}-labeled cells are analyzed immediately for $^{45}Ca^{++}$ content. Quantification of the amount of $^{45}Ca^{++}$ taken up per cell indicate age-induced differences in calcium uptake, whereas matrix calcium content
20   indicates changes in deposition.

          For matrix deposition, equilibrium-labeled cells are allowed to incorporate cellular $^{45}Ca^{++}$ into ECM. Given that both osteocalcin (BGP) and osteonectin bind calcium,
25   this exogenous cell-labeling procedure precludes fluid-phase $^{45}Ca^{++}$ binding to previously synthesized matrix proteins. Subsequently, cells/ECM are removed with (brief) trypsin/EDTA, cells pelleted by centrifugation, and $^{45}Ca^{++}$ incorporation into the
30   trypsin/EDTA extractable ECM determined by scintillation counting. Trypsin-resistant ECM is then removed by Triton X-100 extraction as described previously (Gospodarowicz and Ill, 1980; Long *et al.*, 1990) and counted similarly. To control for residual cell
35   contamination, ECM extracts are monitored for DNA content and calcium deposition into the matrix calculated as a

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total extractable $^{45}Ca^{++}$ corrected for that due to contaminating cells.

     The above calcium loading studies evaluates
5   precisely the uptake and deposition of calcium by human osteoblast cells during their differentiation. The histochemical identification of matrix calcium deposition utilizing the von Kossa staining reaction (Long et al., 1990; Heeley et al., 1973; and Puchtler et al., 1978) is
10   evaluated. The inventors previously correlated the presence of positive von Kossa reactions with the ability of osteoblast cells generated from (unfractionated) precursor cells to metabolically deposit calcium in the extracellular matrix (Long and Dixit, 1990). These
15   cultures are examined for von Kossa reactivity at the same time points of the calcium incorporation/deposition studies. Thus these kinetic studies are correlated with each other, and demonstrates the validity of the von Kossa reaction as an indicator of the early phases of
20   calcification for primary human cells.

Chondrogenic Potential Of Bone Cultures. Bone precursor cells are tested for their functional capacity to undergo chondrogenic differentiation. These studies address an
25   alternative concept that the age-related differentiation blockade may favor chondrogenesis rather than osteogenesis. This is particularly important if the osteoprogenitor cells have chondrogenic potential, a concept often discussed but never definitively proven.
30   However, some suggestion of this exists in a study of rat osteoprogenitor cells (Taniguchi et al., 1993). In this study, peri-osteal injection of TGF-β stimulated neonatal osteoprogenitor cells, whereas in adults it induced differentiation of chondrocytes. The incorporation of
35   $^{35}SO4$ into proteoglycans as well as immunocytochemistry of chondrocytes (as detailed in the Preliminary Results) is used to study chondrogenesis.

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Reddi and co-workers have shown that $^{35}SO_4$ is
incorporated into proteoglycans during the cartilaginous
phase of bone matrix-induced chondrogenesis (Reddi,
1981). Bone cell cultures from both age groups are
5   metabolically labeled with $^{35}SO_4$, and the incorporation
of label into proteoglycan monitored by detection of
chondroitinase ABC (EC 4.2.2.4) - sensitive radiolabel
incorporation. For studies of $^{35}SO_4$ incorporation,
osteoblast cells are differentiated as above, and
10 metabolically labeled (6-12 hrs) with $^{35}SO_4$.
Subsequently, the cells are released and ECM is treated
with chondroitinase ABC and enzymatically-liberated label
monitored by scintillation counting.

15 Bone protein synthesis, alkaline phosphatase
expression, $^{35}SO_4$ incorporation (low), and $^{45}Ca^{++}$
deposition all strongly indicate the presence of
bone-forming cells. However, they do not distinguish
definitively between chondroblasts and osteoblasts. For
20 example, most if not all cells contain surface
proteoglycans, and contaminating cells thus may be
sensitive to chondroitinase release of $^{35}SO_4$. Further,
the demonstration of cellular Ca^{++} deposition while
clearly showing the osteogenic organization of the
25 matrix, does not demonstrate the presence of
hydroxyapatite.

A better indicator of cartilage/bone differences is
the pattern of collagen deposition during bone formation
30 (bone produces type I, and cartilage type II collagen).
These studies employ collagen peptide mapping (via CNBr
cleavage) (Barsh et al., 1980). In this procedure,
collagen synthesis is monitored by metabolic labeling
using ^{3}H-proline (100 Ci/ml; L2,3,4,5 3H proline,
35 Amersham, spec. act 120 Ci/mM). Cells are pre-incubated
in the presence of 50g/ml Na-ascorbate, washed and
metabolically labeled for 16-24 hours. Subsequently, the

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cells are disrupted, lysate dialysed against 1 mM NH_4CO_3, and digested with 100g pepsin in 0.5 M HAc, and subjected to cleavage within the PAGE polyacrylamide gels (Barsh *et al.*, 1980).

5

Several outcomes of the above studies are envisioned. One is that age-related changes might affect bone matrix or bone protein production directly. Conversely, the lack of "normal" bone-ECM or bone protein
10   production by preosteoblasts may actually be due to the "down-regulated" expression of important bone cell markers required as an antecedent to bone ECM mineralization. The studies described in herein distinguish between defects at the level of preosteoblast
15   to osteoblast differentiation, and those alterations occurring in osteoblast function.

The inventors can evaluate human bone precursor cell development in multiple age groups (*e.g.*, 18-25, 26-49
20   and > 50) from the level of the osteoprogenitor cells to the stage of osteoblast cells depositing and calcifying osteoid ECM. Thus, important information is obtained concerning the role of age-induced changes on bone cell differentiation and function, particularly as it relates
25   to calcium metabolism. These data provide a more complete understanding of the events regulating bone cell development in both older and younger individuals.

Example 7:   Osteogenic Genes Which are Differentially
30   **Modified During Aging**

The above examples are designed to determine the nature and degree of the alterations induced by the aging process of human bone precursor cells and their progeny,
35   the osteoblasts. They do not, however, examine the molecular basis of age-induced changes in cellular development. The genetic alterations occurring in these

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cells with aging are determined in this example. The
proliferation and differentiation protocols discussed are
used to evaluate the molecular control of those proteins
specifically altered. Those genes which are uniquely
5   altered in expression during aging are identified.

Analysis Of Bone Protein Messenger RNA. The effects of
age on the regulation of bone protein mRNA (for both
degree of expression, and temporal expression) is
10   analyzed by both Northern analysis and in situ
hybridization. Osteonectin, osteocalcin, alkaline
phosphatase, and osteopontin mRNAs are used as probes for
the matrix molecules. Northern analysis (or the more
sensitive S1 nuclease assay) is performed as described
15   previously by one of the inventors (Long, *et al.*, 1990).

As an alternative, bone precursor cells or
progenitor cell-colonies from both age groups can be
fixed and message expression evaluated by in situ
20   hybridization. This latter procedure is carried out
utilizing biotinylated cDNA probes. Non-isotopic in situ
hybridization is performed as described by Singer *et al.*
(Singer *et al.*, 1986). This procedure yields signal and
noise ratios equivalent to autoradiography while allowing
25   excellent morphological identification (Lawrence *et al.*,
1986). The cDNA probes are prepared utilizing random
primer incorporation of biotinylated ATP (BRL,
Gaithersburg, MD) or other biotinylated dNTPs
(Photobiotin Labeling, BRL).
30

Briefly, paraformaldehyde fixed cells are incubated
in 1% Levisamole to inhibit endogenous alkaline
phosphatase (Schmetzer *et al.*, 1987), and hybridized for
4 hours at 37° C. Following hybridization to cellular
35   RNA, the biotinylated probe is conjugated to
streptavidin, the streptavidin linked to
biotin-conjugated alkaline phosphatase, and reacted with

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bromochloroindolyl phosphate and nibroblue tetrazolium to
yield a blue precipitate at the site of cDNA
hybridization. These studies determine the temporal
expression of know bone protein mRNAs in osteoprogenitor
5   cells (with RT-PCR and in situ hybridization),
preosteoblasts, and osteoblast cells (both with Northern
or S1s). For osteoprogenitor cell colonies, these are
picked at various times during colony-formation (1, 3, 5,
and 7 days) for reverse transcriptase-based polymerase
10   chain reaction (RT-PCR) or are fixed for in situ
hybridization. Preosteoblast and osteoblast cultures
generate sufficient numbers of cells for Northern or S1
analysis.

15   While a variety of methods exist for identifying
differentially expressed genes, most rely on the
subtractive hybridization technique (Liang et al., 1992).
However, such procedures tend to be technically daunting
and prone to experimental error. Recently, Pardee and
20   co-workers have developed a fingerprinting technique for
the comparison of differentially expressed mRNAs (Liang
et al., 1992).

         This procedure, termed Differential Message Display
25   (DMD), allows the separation and rapid cloning of
individual mRNAs by means of the polymerase chain
reaction (PCR). DMD utilizes a set of oligonucleotide
primers in which the 3' primer is anchored to the
polyadenylate tail of this subset of RNAs, and the
30   5' primer is a short, arbitrary sequence. Following
reverse transcriptase-based generation of cDNAs, the mRNA
populations defined by these primers are amplified by
PCR, in the presence of a radio-labeled dNTP, and
resolved on a DNA sequencing gel. Thus, using a number
35   of differing primer-pairs results in detection of
differentially expressed messages (e.g., in younger
versus older bone cell DMD age comparisons).

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Subsequently, it was shown that the number of anchored
primers could be reduced from twelve to four by using a
degenerate base at the penultimate 3' end (Liang *et al.*,
1994).

5

In another embodiment, genetic alterations
associated with the aging process, or disease, can be
determined by screening cDNA libraries from elderly
individuals. In this procedure, a cDNA library from
10   elderly individuals (of either or both antigenically
different groups described above) are evaluated for
expressed genes that are not found in younger
individuals.

15   To accomplish this, human bone precursor cells are
purified from each age group (*e.g.*, <25 years, and >60
years) and cDNA libraries from each constructed. cDNAs
from the younger individuals are pooled, radiolabeled,
and used to screen the cDNA library obtained from the
20   human bone precursor cells of the most elderly
individuals. Those cDNAs in common between the elderly
and younger individuals will hybridize with each other
(thus being detected by autoradiography), whereas those
unique to the elderly, will not. These latter cDNAs
25   represent genes not expressed in younger individuals.

Likewise, using radiolabeled cDNA from elderly
individuals, cDNA libraries from young individuals can be
screened for the presence of genes only expressed in bone
30   cells from younger individuals (hence being genes the
expression of which is lost during aging). Other
comparison envisioned, but not limited to, are contrasts
between young, old, and middle aged individuals, or
contrasts of different types of osteoporosis and bone
35   disease with healthy individuals.

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Finally, the inventors demonstrated that the molecular basis of the differences in normal versus cancer cells could be easily visualized by DMD (Liang et al., 1992). The inventors utilize DMD to identify genes
5 uniquely modified by aging. In these investigations preosteoblasts and osteoblasts from both age groups are evaluated by DMD. Differentially expressed gene fragments are directly sub-cloned from the PCR products (into the pCR1000 vector, Invitrogen), and used as probes
10 for isolating the full length cDNA from appropriate libraries. Moreover, as most subcloned fragments are 400-1000 bp, sequencing and gene bank searches identify that sequence or its homology with other genes.

15 The significance of the mRNA studies is that the increase the inventors' understanding of the regulation of bone protein expression during aging. Comparison of this data determines the level of regulation (i.e. transcription of gene expression or translation into
20 protein), thus linking biochemical control mechanisms to molecular control of gene expression. The mRNA studies confirms immunological-based observations on bone protein expression and extend these data to the RNA level.

25 The inventors propose isotopic RNA analysis for its sensitivity, and non-isotopic in situ labeling for ease of use, preservation of morphology, and permanence of record. The comparison of Northern (or S1) analysis with in situ hybridization will allow exact evaluation amount
30 of mRNA, as well as identification of the cell types expressing a given message. One problem with in situ hybridization is that it only detects mRNA of relatively abundant copy number. Thus low-copy numbers of bone protein mRNA may go undetected. To overcome this
35 limitation RT-PCR is utilized. Using antisense RNA primers, reverse transcriptase, and 30 cycles of PCR, low copy mRNA signals can be amplified up to 109 fold.

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Example 8:   The Determinants of Human Bone Precursor
                 Cell Proliferation in Low-Shear
                 Environments

5   The physical requirements for optimal bone precursor
cell (i.e., osteoprogenitor cell and pre-osteoblast)
proliferation both in vivo and ex vivo are poorly
understood. In vivo, bone formation most often occurs
within an intervening cartilage model (i.e., endochondral
10   ossification). This well-understood bone histogenesis is
one of embryonic and post-natal chondrogenesis, which
accounts for the shape of bone, and the subsequent
modification and calcification of bone cell ECM by
osteoblasts. This process results in the formation and
15   elongation of bone during childhood growth and
development.

        However, there is no evidence that adult trabecular
bone formation (the predominant site of calcium
20   metabolism) occurs in an identical fashion. For example,
in traumatic bone repair, mesenchymal cells migrate into
and organize the hematoma which occurs around a fracture.
As a result, a large irregular-shaped bony callus
(comprised of woven bone) forms, that subsequently is
25   remodeled into the shape of the original bone. A role
for chondrocytes in this process has not been described.
Other evidence (from this group) supports the concept
that trabecular bone formation is different from the
classic endochondral model.
30
        As described above, trabecular bone precursor cells
proliferate and organize their extracellular matrix in an
osteogenic fashion - without an apparent intervening
cartilage model. The inventors have shown that
35   trabecular bone differs from compact bone in its
extracellular matrix protein composition, suggesting
differences in trabecular osteoblast function (Ninomyia

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et al. 1990). Nonetheless, it is likely that trabecular bone formation in the adult requires a three-dimensional (osteoid) matrix that is elaborated by the osteoblasts themselves. Moreover, the inventors have demonstrated

5   that osteoprogenitor cell growth in the presence of a three-dimensional fibrin clot (interestingly, one of the major proteins in a hematoma) results in a marked increase in: progenitor cell cellularity, the expression of bone protein antigenic markers, cell size, and cell

10 complexity.

Human bone precursor cell proliferation is likely to be augmented by growth in simulated microgravity environments. This is based on bone precursor cell

15 proliferation from differentiation in rat space-flight studies discussed above (Klement and Spooner, 1993). In addition, Goodwin and associates have documented a remarkable augmentation of cell proliferation in low-shear rotating wall vessle (RWV)-type bioreactors. Their

20 data shows that mesenchymal cell types show an average three to six-fold increase in cell density in these bioreactors reaching a cellularity of approximately 10^7 cells/mL. Importantly, this increase in cell density was associated with a 75% reduction in glucose utilization as

25 well as an approximate 85% reduction in the enzymatic markers of anabolic cellular metabolism (SGOT and LDH). Further work by Goodwin *et al.* shows that the growth of mesenchyml cells (kidney and chondrocyte) under low-shear conditions leads to the formation of tissue-like cell

30 aggregates which is enhanced by growing these cells on collagen-coated microcarriers.

Physical Requirements for Bone Precursor Cell Proliferation. The physical requirements for bone

35 precursor cell growth in rotating wall vessels is evaluated by comparing suspension-phase and microcarrier-based cell growth. Four bone cell culture conditions are

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utilized. The two types of simulated microgravity
cultures consist of suspension-phase bone precursor cells
only, and simulated microgravity cultures with precursor
cells plus microcarrier beads. These cultures are
5   contrasted with control cells cultured in unit-gravity
conditions (unit-gravity cultures are performed in tissue
culture flasks of equal volume and media-composition;
i.e., both cells in suspension and cells cultured on
microcarriers). Interestingly, the inventors have
10   recently demonstrated that unit-gravity bone precursor
cell growth occurs in type I collagen gels. Collagen-
coated microcarrier (Cytodex-3 beads) as described above
in the inventors' studies are utilized.

15   These studies evaluate whether human bone precursor
cells require cell:cell interaction (as evident in
suspension phase comparisons of simulated-microgravity
and unit gravity cultures). Whether human bone precursor
cells may require both cell:cell contact and/or cell:ECM
20   interactions is determined. This possibility is
determined in microcarrier-based studies in which cells
both interact with each other, and with the type I
collagen coat of the carrier beads. It is understood
that while human bone precursor cells may self-assemble
25   into aggregates in simulated-microgravity suspension-
phase cultures, other cell types (lymphocytes and
lymphocyte cell lines) show reduced proliferation.
However, these are hematopoietic cells which do not
generate a solid tissue, such as cartilage or bone.
30

Quantitative Assessment of Human Bone Precursor Cell
Development. Human bone precursor cells are cultured for
varying periods under simulated-microgravity conditions.
Based on the developmental periods utilized to generate
35   the bone cell data, bone cell development in the RWVs on
days three, five, seven, nine, twelve, fourteen, and
thirty are evaluated. Total cellularity is evaluated by

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cell counts, cell-density determination, and evaluation
of the frequency of tissue-like aggregates in these
cultures. For microcarrier cultures, aggregates are
enumerated and the cells treated to remove them from the
5   microcarrier beads. Cultures are stripped of cells by
the method of Gospodarowicz, (Gospodarowicz and Ill,
1980; Gospodarowicz et al., 1980) which leaves behind
cell-derived extracellular matrix (ECM) and/or beads.
Also, previous work has shown that this procedure easily
10   releases mesenchymal cells from cytodex beads, and does
not interfere with bone antigen expression (Long et al,
1995).

Multiparameter Flow Cytometry Characterization of
15   *Preosteoblasts and Osteoblasts*. Single-cell suspensions,
taken at specified time-points, are evaluated for
developmental markers by multiparameter flow cytometry.
Two and three color fluorescence-activated flow cytometry
is used to evaluate co-expression of a number of human
20   bone protein markers: osteonectin, osteocalcin, alkaline
phosphatase, bone sialoprotein, and osteopontin. The
expression of these proteins is also correlated with
alterations in cell size (as indicated by forward angle
light scatter) and cell complexity (as indicated by right
25   angle light scatter). These studies allow for the
evaluation of quantitative differences in both the
phenotype and frequency of pre-osteoblasts and
osteoblasts as defined by their flow cytometry
characteristics.
30
Osteoprogenitor Cells. Alterations in the frequency of
osteoprogenitor cells during simulated microgravity
exposure is evaluated. At the times indicated above,
samples of cells are removed from the cultures and
35   osteoprogenitor cell numbers are evaluated in fibrin-clot
colony-forming assays. These studies determine whether
the frequency or proliferative potential of the two

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classes of osteoprogenitor cell (high proliferating
colony-forming progenitor *vs*. low proliferating, cluster-
forming progenitor cells) are differentially modulated
under low-shear conditions. Evaluation of the colony
5   cellularity (cells/colony) also determines the
proliferative potential of each of these progenitor cell
phenotypes.

As mentioned, evidence for a differentiation
10   blockade was noted during real microgravity exposure
(Klement and Spooner, 1993). However, the exact level of
this blockade is unknown. With the inventors' ability to
quantify bone precursor cells, the inventors can locate
the level at which such a block occurs for human cells.
15   For example, if a blockade exists between the
osteoprogenitor cell and pre-osteoblast then numbers of
the former should increase in frequency with an attendant
decrease in a pre-osteoblast numbers. A similar
alteration in cell ratios is detectable if the blockade
20   is between the pre-osteoblast and osteoblast.

*Biochemical and Metabolic Evaluation of Bone Cell
Development*. In order to evaluate the metabolic status
of developing bone precursor cells, simulated
25   microgravity and control cultures are monitored for
alterations in glucose utilization, SGOT and LDH enzyme
production as described previously (Goodwin *et al*.,
1993). These studies yield information about the
efficiency of glucose utilization, gluconeogenesis, and
30   level of cellular damage.

Alterations in the cellular expression of bone
proteins as evaluated by flow cytometry (above) by two
alternative methods. Bone protein production of both
35   collagenous and non-collagenous proteins are monitored by
metabolic (^{35}S-methionine) labeling and subsequent
immunoprecipitation of the relevant proteins as described

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by (Greenberg *et al.*, 1990; Kozawa *et al.*, 1989; Tracey *et al.*, 1990). Bone protein quantity is monitored using a number of independent assays. Secretory bone protein release is monitored by soluble-phase RIA. Cellular

5   protein synthesis is monitored by immunoprecipitation of metabolically labeled cells.

Briefly, human bone precursor cells are cultured for various periods as described above, and culture media

10   exchanged for methionine-free media (Gibco) containing 10 μCi of ^{35}S-methionine (4 hours at 37°C). Cells are washed free of unincorporated label, lysed with triton X-100 and proteins immunoprecipitated with cold TCA. The proteins in the final precipitate are resolved by PAGE

15   and visualized by autoradiography. ^{125}I-labeled purified bone proteins are run as controls. These protein synthesis studies are correlated with immunocytochemical analysis, performed using an avidin-biotin peroxidase labeled system as described by Long *et al.* (Long and

20   Heffner, 1988; Long and Dixit, 1990).

In addition, information on the micro-structure of bone cell aggregates is determined by ultrastructural analysis. Both scanning electron microscopy and

25   transmission electron microscopy are employed to analyze the quality and degree of organization occurring within bone tissue-like aggregates as previously described.

Example 9:   **Cytokine and ECM Control of Bone Precursor**
30   **Cell Proliferation in Low-Shere**
                     Microenvironments

Once the optimal physical/geometrical requirements for human bone precursor cell expansion in simulated

35   microgravity are determined, the mechanism and/or further expansion of these cells by various osteogenic cytokines is determined. Certain growth factors (or cytokines), in

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particular TGF-β1 and bFGF, stimulate the early phases of
osteoprogenitor cell proliferation. One potential
mechanism of increased *ex vivo* expansion of bone
precursor cells in simulated microgravity is altered
5   responsiveness to mitogenic activation.

As discussed above, glucose metabolism decreases
while cell density and tissue aggregate formation
increases under simulated microgravity conditions
10   (Goodwin *et al*, 1993). One reason for this is that
increased availability of growth factors and nutrients
occurs due to coriolis-force driven admixing within the
rotating wall vessel. A correlate of this is that the
response of these cells is seemingly heightened at a
15   given concentration of growth factor (i.e. due to
improved availability).

The role of differing cytokines in the expansion of
bone precursor cells in simulated microgravity is
20   obtained. Cultures with varying concentrations of
osteogenic growth factors are initiated. Given their
effect on proliferation, TGF-β and bFGF, both alone and
in combination are tested. Comparison of cell growth in
simulated microgravity and unit gravity cultures utilize
25   all the quantitative methods described above. The
inventors also documented differential effects for
various cytokines at the early and late phases of bone
precursor cell proliferation (e.g., TGF-β, and 25-
dihydroxy vitamin D_3, respectively). A synergistic
30   interaction likely occurs if bone precursor cells are
cultured simultaneously with these two classes of growth
factors under simulated microgravity conditions. The
inventors evaluate four relevant combinations of growth
factors: TGF-β + BMP; TGF-β + D_3; bFGF + BMP and bFGF +
35   D_3. Quantitative evaluation of these combinations is
performed as outlined above.

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Another component of the osteogenic microenvironment
is the extracellular matrix. As mentioned, bone
extracellular matrix contains both collagenous and non-
collagenous proteins. When bone precursor cells are
5   cultured on certain non-collagenous proteins, they show
an increase in proliferation in bone cell expression.
Moreover, the applicants have shown, using the
hematopoietic system as a model, that subpopulations of
primitive progenitor cells require both a mitogenic
10   cytokine and a specific extracellular matrix (ECM)
molecule in order to proliferate. (Long *et al.*, 1992)
Indeed, without this obligate matrix:cytokine
(matricrine) signal, the most primitive of blood
precursor cells fail to develop *in vitro* (Long *et al.*,
15   1992). It is likely that a similar requirement exists
for human bone precursor cells. Further it is likely
that osteogenic development in simulated microgravity may
require the addition of exogenous ECM molecules.

20   The inventors have demonstrated the importance of
three bone ECM proteins in human bone cell growth at unit
gravity: osteonectin, osteocalcin, and type I collagen
(Long and Ashcraft, 1994). Two additional matrix
proteins are of importance: bone sialoprotein and
25   osteopontin (Oldberg *et al.*, 1986; Nomura *et al.*, 1988).
These two proteins are believed to be involved in bone
formation but their role is unknown.

The function of these ECM molecules, soluble,
30   purified bone ECM proteins are investigated in simulated
microgravity and control cultures. Varying amounts of
exogenous ECM molecules is added in the presence of a
single growth factor (e.g., 25 pM TGF-β, or the optimal
factor/concentration defined for simulated microgravity
35   environments as detailed above), to allow precise
evaluation of the developmental effects of each
extracellular matrix molecule. The combined effects of

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relevant cytokine and matrix molecules on the expansion
of human bone precursor cells in simulated microgravity
is determined. Each of the effective cytokines in matrix
molecules defined are added at optimal concentrations and
5   cell proliferation evaluated as detailed above.

The above studies show, in a stepwise fashion, (1)
the correct physical/geometrical requirement for bone
progenitor precursor cell growth in simulated
10   microgravity, (2) the appropriate cytokine or cytokine
combinations, and (3) the relevant ECM molecules
necessary for human bone precursor cell proliferation. A
complex osteogenic microenvironment therefore is reduced
to a stepwise evaluation and optimalization of each of
15   its relevant components. These studies define the
minimum essential conditions for the *ex vivo* expansion of
human bone precursor cells in simulated microgravity
environments.

20   **Example 10:   The Responsiveness of Developing Bone
                        Precursor Cells to Other
                        Microenvironmental Signals in Simulated-
                        Microgravity.**

25   Developing tissue cells interact with a wide variety
of regulators during their ontogeny. Thus, cells
interact with each other, with growth factors, and with
extracellular matrix molecules. Each of these
interactions is mediated by defined, specific receptor-
30   ligand interactions necessary to both stimulate the cell
proliferation and/or motility. Both chemical and/or
extracellular matrix gradients exist which signal the
cell to move into a defined microenvironment. As well,
high concentrations of the attractant, or other signals,
35   next serve to "localize" the cell, thus stopping its non-
random walk. Signals which stop and/or regionalize cells
in appropriate microenvironments are poorly understood.

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The inventors have shown that, in the hematopoietic
system, complexes of cytokines and extracellular matrix
molecules serve to localize progenitor cells (Long *et al.*
1992). It is likely that similar mobility (chemotactic)
5   or localization signals exist for bone precursor cells,
and mediate their movement into an osteogenic region
(such as a fracture). The modulation of these
physiological processes in simulated microgravity
conditions is investigated.
10

One Precursor Cell Cytoadhesion and Tissue Localization.
Recently, the inventors have developed a cytoadhesion
assay which employs "caged" fluorochromes to label
isolated progenitor cells for subsequent adhesion
15   studies. In this assay, acetylmethylester derivatives of
FITC are used to viably label the cells. Upon
internalization, intracellular esterases cleave the AM-
ester derivative rendering the released fluorochrome
relatively impermeable. Importantly, the fluorescence
20   signal is linear with respect to cell number, and as few
as several hundred cells can be detected (FIG. 10A). The
cytoadhesion assay then consists of the adhesion of
caged-fluorochrome labeled cells to purified and/or
recombinant proteins which are immobilized onto tissue
25   culture plastic (as described previously (Long and Dixit,
1990; Long *et al.*, 1992)), the removal of non-adherent
cells, and quantitation in a fluorescent plate reader.

The resultant sensitivity of this assay is
30   approximately 100 times greater than other cytoadhesion
assays reported previously from this laboratory (Long *et
al.*, 1992; Long and Dixit, 1990; Campbell *et al.*, 1990).
The inventors utilized this assay in preliminary studies
of purified human bone precursor cells to evaluate
35   attachment to extracellular matrix molecules. These data
indicate that bone precursor cells express differential
attachment capacities to both immobilized bone ECM

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molecules and immobilized cytokines (FIG. 10B). Previous
work showed that hematopoietic progenitor cells bound to
both growth factors and ECM molecules (Long et al., 1992;
Long and Dixit, 1990; Campbell et al., 1990).

5

Thus, as divergent cellular phenotypes as bone and
hematopoietic cells both demonstrate dual requirements
for matrix and cytokine molecules in the localization
(adhesion) process. Notably, the binding of progenitor
10   cells to immobilized, solitary cytokines further
demonstrates that the presence of growth factors (which
are often themselves immobilized within the extracellular
matrix (Long, 1992)) is as least partially responsible
for the lineage-specific localization of cells.
15   Nonetheless, it is likely that the presence of specific
ECM molecules strengthens this localization process.

In order to evaluate the role of cytoadhesion in
bone precursor cell proliferation, the unique aspects of
20   a simulated microgravity in which adhesive interactions
may be reduced (in the case of a simulated microgravity
tissue culture in the absence of microcarriers) or
differentially augmented (with microcarriers) is
utilized. To perform these studies, cultures of
25   proliferating bone precursor cells are established under
optimal cytokine conditions as defined above, or (as a
starting point) as defined in unit-gravity conditions.
The capacity of simulated microgravity cultured cells to
interact (adhere) with both bone cell regulatory
30   cytokines (bFGF, TGF-β1, and BMP-2) and extracellular
molecules (osteonectin, osteocalcin, bone sialoprotein,
osteopontin, fibronectin and thrombospondin) is
evaluated. The latter two proteins (thrombospondin and
fibronectin) are present in bone extracellular matrix,
35   and are important as cytoadhesion molecules in developing
tissues (Weiss and Reddi, 1980; Clezardin et al., 1989).

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Bone Precursor Cell Chemotaxis. In a likewise fashion,
the effects of simulated microgravity on the motility
machinery of developing bone precursor cells is
investigated. Various bone related growth factors are
5   evaluated for their capacity to direct non-random
movement (cytokinesis) and non-random migration
(chemotaxis). As mentioned, early bone precursor cells
possess the ability to actively migrate into the area of
bone injury, there differentiating into bone-forming
10   cells. However, no information exists on the factors or
events which signal this important migratory process.

Simulated microgravity-cultured and control cells
are evaluated for responsiveness to both known
15   chemotactic factors (chemokines; i.e., interleukin-8,
GM-CSF, M-CSF) and for the role of osteogenic growth
factors in stimulating either chemokinesis or chemotaxis.
In particular, bFGF and TGF-β1, both powerful regulators
of bone progenitor cell proliferation are evaluated. In
20   order to evaluate the effects of microgravity on bone
precursor cell migratory capabilities, a panel of known
leukocyte chemotactic factors and osteogenic factors in
direct comparison to unit gravity control cells is used.

25   The chemokines are members of a chemotactic cytokine
supergene family (Oppenheim *et al.*, 1991). The
chemokine-α cytokines are comprised of molecules with
their first two cysteines interrupted by an amino acid
(C-X-C), and are represented by such molecules as
30   interleukin-8 (IL-8) and platelet factor 4 (PF4). MCP-1
and RANTES are representative of the chemokine-β
subfamily, and are characterized by an uninterrupted C-C
arrangement. The use of IL-8 and MCP-1 allows employment
of chemotactic factors which are known to induce
35   migration of a broad spectrum of cells (Oppenheim *et al.*,
1991).

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Example 11:   The Ability of Bone Precursor Cells to
                 Differentiate Into Osteoblast Cells or
                 Affect the Capacity of Osteoblasts to
                 Elaborate an Osteoid Extracellular Matrix
5   in Simulated Microgravity

     The complete evaluation of bone cell development
requires the study of both bone precursor cells
(osteoprogenitor cells and pre-osteoblasts) as well as
10   their differentiated progeny, the osteoblasts. It is the
osteoblasts that are responsible for the elaboration and
mineralization of bone matrix, and the subsequent
formation of bone. As mentioned, studies of space-flight
and (bed-rest) models of weightlessness indicate that the
15   differentiated function of osteoblasts is compromised in
microgravity conditions. As a result, there is a calcium
and bone loss, in both periosteal, and trabecular
regions, with the latter predominating.

20   On a cellular level, osteoblasts reduce or lose
their capacity to produce osteoid matrix (Klement and
Spooner, 1993) with little alteration occurring in
osteoclast activity. Finally, microgravity induces a
disassociation between bone precursor cell proliferation
25   and differentiation, that results in a differentiation
blockade causing an increased number of precursor cells
relative to the numbers of differentiated osteoblasts
(Klement and Spooner, 1993).

30   Most of the quantitative data on microgravity-
induced bone loss are from whole-animal studies, and tend
to be predominantly morphometric and retrospective in
nature. Thus, the site of biochemical/molecular defects
leading to bone loss can only be identified in simulated
35   microgravity studies. Importantly, such studies cannot
be performed on trabecular bone outgrowth cultures for a
variety of reasons (limiting cell numbers, lack of

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purified cells, heterogeneity of cell types present,
etc.), and because the principle defect may be a failure
of the osteoblast differentiation pathway. That is, cell
development may not progress from osteoprogenitor cell to
5   pre-osteoblast, or from pre-osteoblast to osteoblast.
Therefore, it is best to examine this problem in a system
in which the full spectrum of human bone cell
proliferation/differentiation can be evaluated.

10   *Regulation of Osteoblast Cell Differentiation.* As
described herein, the inventors have developed a system
in which isolated and purified bone precursor cells
proliferate and differentiate into osteoblast cells. In
particular, the inventors have demonstrated that the
15   process of switching serum-free cultures to TGF-β-driven,
serum-containing cultures results in a differentiation of
pre-osteoblasts into osteoblast cells. Importantly, the
osteoblast cells generate an osteoid extracellular matrix
in which non-collagenous bone proteins are deposited and
20   the cells calcify the extracellular matrix.

The effect of simulated microgravity on both the
differentiation of pre-osteoblasts into osteoblasts, and
the subsequent alterations in differentiated cell
25   (osteoblasts) function is evaluated. As such, the cell
culture protocol described above is modified to induce
osteoblast cell differentiation. In these studies,
simulated microgravity cultures of human bone precursor
cells will are run in two phases. The first phase (7-10
30   days) is an *ex vivo* expansion step utilizing serum-free
cultures containing growth factor/matrix combinations (as
defined above) for generation of maximum numbers of bone
precursor cells.

35   During this phase of the cultures, only cellularity
and aggregate formation is monitored as an index of
culture conditions. At optimal cell density, these

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cultures are switched to serum-containing, TGF-β-
containing conditions. Subsequent studies (over an
additional 7-10 days) evaluate osteoblast cell
differentiation patterns and functional capacities.

5

As described herein, the inventors have defined a
multi-parameter flow cytometric analysis of osteoblast
cell differentiation markers; these cells increase in
cell size, cell complexity, and the expression of
10   multiple markers of bone cell differentiation. The
inventors evaluate the capacity of pre-osteoblasts to
differentiate into osteoblast cells using this multi-
parameter flow cytometric analysis.

15   In particular, the expression of the bone proteins
discussed above plus two additional markers of bone
differentiation, alkaline phosphatase and type I collagen
are evaluated. Alkaline phosphatase (EC 3.1.3.1) are
monitored by both flow cytometry as well as by alkaline
20   phosphatase cytochemistry (Reddi, 1981). This latter
procedure distinguishes bone alkaline phosphatase from
that of liver based on heat and urea sensitivity. The
production and deposition of bone extracellular matrix
molecules microgravity-derived osteoblast cells is
25   determined by metabolic labeling and immunoprecipitation
using ^{35}S-methionine immunoprecipitation.

Extracellular Matrix Calcification. Studies on the
capacity of simulated microgravity and control culture
30   osteoblast cells to mineralize their surrounding (bone)
ECM employ two alternative approaches: metabolic labeling
with ^{45}Ca^{++} and histochemical analysis utilizing the von
Kossa staining procedure. For the calcium labeling
studies, two aspects of bone cell calcium metabolism are
35   evaluated: their ability to take up calcium and their
ability to deposit calcium into the extracellular matrix.

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In these studies, simulated microgravity-expanded
osteoblasts are removed from the rotating wall vessel and
equilibrium-labeled (briefly, one hour) with $^{45}Ca^{++}$, as
described above. Briefly, osteoblasts differentiated in
5   simulated microgravity (on days 3, 5, 7, 14 and 21 post
serum-stimulation) are removed and washed 3 times with
calcium-free PBS, and metabolically labeled with 50 μCi
$^{45}Ca^{++}$ (as $CaCl_2$; Amersham, Arlington Heights, IL;
sp.act. 733 mBq/Mg) for 60 min at 37°C. Following $^{45}Ca^{++}$
10   calcium-equilibration, labeled cells are washed free of
unincorporated calcium, resuspended in serum-free tissue
culture medium and re-established in the original culture
dishes or in simulated-microgravity conditions. An
aliquot of Ca^{++}-labeled cells are analyzed immediately
15   for $^{45}Ca^{++}$ content. Quantification of the amount of
$^{45}Ca^{++}$ taken up per cell indicate microgravity-induced
differences in calcium uptake, whereas matrix calcium
indicates deposition.

20   For matrix deposition, equibrium-labeled cells are
allowed to incorporate cellular $^{45}Ca^{++}$ into ECM. Given
that both osteocalcin (BGP) and osteonectin bind calcium,
this exogenous cell-labeling procedure precludes fluid-
phase $^{45}Ca^{++}$ binding to previously synthesized matrix
25   proteins. Subsequently, cells/ECM are removed with
trypsin/EDTA), cells pelleted by centrifugation, and
$^{45}Ca^{++}$ incorporation into the trypsin/EDTA extractable
ECM determined by scintillation counting. Trypsin-
resistant ECM is removed by Triton X-100 extraction as
30   described previously (Gospodarowicz and Ill, 1980;
Gospodarowicz et al., 1980) and counted similarly. To
control for residual cell contamination, extracts are
monitored for DNA content and calcium deposition into the
matrix calculated as a total extractable $^{45}Ca^{++}$ corrected
35   for that due to contaminating cells.

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The calcium loading studies evaluate, precisely, the uptake and deposition of calcium by human osteoblast cells during their differentiation in simulated microgravity and unit gravity conditions. The

5   histochemical identification of matrix calcium deposition utilizing the von Kossa staining reaction is also evaluated (Heeley and Irving, 1973; Puchtler and Meloan, 1978). The inventors previously correlated the presence of positive von Kossa reactions with the ability of

10   osteoblast cells generated at unit gravity to metabolically deposit calcium in the extracellular matrix. As a prelude to actual space-flight studies, simulated microgravity and control cultures for von Kossa reactivity at the same time points of the calcium

15   incorporation/deposition studies is evaluated. These kinetic studies are correlated with each other, and should further demonstrate the validity of von Kossa reaction as an indicator of calcification in simulated microgravity conditions.

20

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have

25   been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and

30   scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and

35   modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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5   supplementary to those set forth herein, are specifically
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CLAIMS

1.   A process for preparing an enriched population of
bone precursor cells, comprising the steps of:

5

        obtaining a population of cells that includes bone
            precursor cells;

        enriching said population of cells for bone-
10   precursor cells by exposing said cells to a
            bone precursor cell antibody immunoreactive
            with a bone precursor cell antigen; and

        removing cells of the population that do not
15   immunoreact with said antibody.

2.   The process of claim 1, wherein said population of
cells that includes bone precursor cells is a population
20   of bone marrow cells.

3.   The process of claim 1, wherein said population of
cells that includes bone precursor cells is a population
25   of peripheral blood cells.

4.   The process of claim 1, further comprising
equilibrium-density centrifugation of said population of
30   cells to provide cells with a density of between about
1.050 and about 1.090 grams/cm^3.

5.   The process of claim 4, further comprising
35   equilibrium-density centrifugation of said population of
cells to provide cells with a density of between about
1.060 and about 1.085 grams/cm^3.

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6.   The process of claim 1, further comprising removing adherent stromal cells by exposing said population of cells to an adherent surface.

5

7.   The process of claim 6, wherein said adherent surface is tissue culture plastic or tissue culture glass.

10

8.   The process of claim 1, further comprising fractionating said enriched population of bone precursor cells according to cell size to obtain cells with an average diameter of between about 8 microns and about 70

15   microns.

9.   The process of claim 8, further comprising fractionating said enriched population of bone precursor

20   cells according to cell size to obtain cells with an average diameter of between about 10 microns and about 20 microns.

25   10.   The process of claim 1, further comprising fractionating said enriched population of bone precursor cells according to cell size by fluorescence activated flow cytometry, velocity sedimentation, or counter-flow centrifugal elutriation.

30

11.   The process of claim 1, wherein the enrichment of bone precursor cells is between about 100-fold and about 1,000-fold over said population of cells.

35

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12.   The process of claim 1, wherein the enrichment of bone precursor cells is between about 1,000-fold and about 2,000-fold over said population of cells.

5

13.   The process of claim 1, wherein the enrichment of bone precursor cells is between about 2,000-fold and about 3,000-fold over said population of cells.

10

14.   The process of claim 1, wherein the enrichment of bone precursor cells is between about 3,000-fold and about 4,000-fold over said population of cells.

15

15.   The process of claim 1, wherein the enrichment of bone precursor cells is about 4,800-fold over said population of cells.

20

16.   The process of claim 1, wherein said population of cells that includes bone precursor cells is exposed to an anti-osteocalcin, anti-osteonectin or anti-bone alkaline phosphatase antibody.

25

17.   The process of claim 16, wherein said population of cells that includes bone precursor cells is exposed to an anti-osteocalcin antibody.

30

18.   The process of claim 16, wherein said population of cells that includes bone precursor cells is exposed to an anti-osteonectin antibody.

35

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19.   The process of claim 16, wherein said population of
cells that includes bone precursor cells is exposed to an
anti-osteocalcin antibody and to an anti-osteonectin
antibody.

5

20.   The process of claim 19, wherein said population of
cells that includes bone precursor cells is exposed to an
anti-osteocalcin antibody, an anti-osteonectin antibody

10   and to an anti-bone alkaline phosphatase antibody.

21.   The process of claim 1, wherein said population of
cells that includes bone precursor cells is a mammalian

15   cell population.

22.   The process of claim 21, wherein said population of
cells that includes bone precursor cells is a human cell

20   population.

23.   The process of claim 1, wherein said bone precursor
cell antibody is conjugated to a solid substrate.

25

24.   The process of claim 23, wherein said bone precursor
cell antibody is conjugated to a plastic surface, glass
surface, agarose, acrylamide, lectin, or a magnetic

30   particle.

25.   The process of claim 24, wherein said bone precursor
cell antibody is conjugated to a magnetic particle.

35

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26.   The process of claim 1, further comprising exposing
said population of cells immunoreacted with said bone
precursor cell antibody to a second antibody that is
immunoreactive with said bone-precursor cell antibody,
5   and removing cells of the population that do not
immunoreact with said bone precursor cell antibody.

27.   The process of claim 26, wherein said second
10   antibody is conjugated to a solid substrate.

28.   The process of claim 27, wherein said second
antibody is conjugated to a plastic surface, glass
15   surface, agarose, polyacrylamide, lectin, or a magnetic
particle.

29.   The process of claim 1, wherein said bone precursor
20   cells express osteocalcin, osteonectin, or bone alkaline
phosphatase but do not express the pan-hematopoietic
antigen CD34.

25   30.   The process of claim 1, wherein said bone precursor
cells are osteoprogenitor cells.

31.   The process of claim 1, wherein said bone precursor
30   cells are preosteoblast cells.

32.   An enriched population of bone precursor cells
preparable by a process comprising the steps of:
35

        obtaining a population of cells that include bone
              precursor cells;

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enriching the population of said bone-precursor
    cells by exposing said cells to a bone
    precursor cell antibody immunoreactive with a
    bone precursor cell antigen; and

5

removing cells of the population that do not
    immunoreact with said antibody.

10   33.   The cell population of claim 32, wherein said
process comprises obtaining a population of bone marrow
cells.

15   34.   The cell population of claim 32, wherein said
process comprises obtaining a population of peripheral
blood cells.

20   35.   The cell population of claim 32, wherein said
process further comprises equilibrium-density
centrifugation of said population of cells to provide
cells with a density of between about 1.050 and about
1.090 grams/cm^3.

25

36.   The cell population of claim 35, wherein said
process further comprises equilibrium-density
centrifugation of said population of cells to provide
30   cells with a density of between about 1.060 and about
1.085 grams/cm^3.

37.   The cell population of claim 32, wherein said
35   process further comprises removing adherent stromal cells
by exposing said population of cells to an adherent
surface.

38.   The cell population of claim 37, wherein said
adherent surface is tissue culture plastic or tissue
culture glass.

5

39.   The cell population of claim 32, wherein said
process further comprises fractionating said enriched
population of bone precursor cells according to cell size
to obtain cells with an average diameter of between about
10   8 microns and about 70 microns.

40.   The cell population of claim 39, wherein said
process further comprises fractionating said enriched
15   population of bone precursor cells according to cell size
to obtain cells with an average diameter of between about
10 microns and about 20 microns.

20   41.   The cell population of claim 32, wherein said
process further comprises fractionating said enriched
population of bone precursor cells according to cell size
by fluorescence activated flow cytometry.

25

42.   The cell population of claim 32, wherein the
enrichment of bone precursor cells is between about
100-fold and about 1,000-fold over said population of
cells.
30

43.   The cell population of claim 32, wherein the
enrichment of bone precursor cells is between about
1,000-fold and about 2,000-fold over said population of
35   cells.

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44.   The cell population of claim 32, wherein the
enrichment of bone precursor cells is between about
2,000-fold and about 3,000-fold over said population of
cells.

5

45.   The cell population of claim 32, wherein the
enrichment of bone precursor cells is between about
3,000-fold and about 4,000-fold over said population of
10   cells.

46.   The cell population of claim 32, wherein the
enrichment of bone precursor cells is about 4,800-fold
15   over said population of cells.

47.   The cell population of claim 32, wherein said
process comprises exposing said population of cells that
20   includes bone precursor cells to an anti-osteocalcin
antibody or anti-osteonectin antibody.

48.   The cell population of claim 47, wherein said
25   process comprises exposing said population of cells that
includes bone precursor cells to an anti-osteocalcin
antibody.

30   49.   The cell population of claim 47, wherein said
process comprises exposing said population of cells that
includes bone precursor cells to an anti-osteonectin
antibody.

35

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50. The cell population of claim 47, wherein said process comprises exposing said population of cells that includes bone precursor cells to an anti-osteonectin antibody and an anti-osteocalcin antibody.

5

51. The cell population of claim 32, wherein said population of cells that includes bone precursor cells is a mammalian cell population.

10

52. The cell population of claim 51, wherein said population of cells that includes bone precursor cells is a human cell population.

15

53. The cell population of claim 32, wherein said bone precursor cell antibody is conjugated to a solid substrate.

20

54. The cell population of claim 53, wherein said bone precursor cell antibody is conjugated to a plastic surface, glass surface, agarose, polyacrylamide, or a

25 magnetic particle.

55. The cell population of claim 54, wherein said bone precursor cell antibody is conjugated to a magnetic

30 particle.

56. The cell population of claim 32, further comprising exposing said population of cells immunoreacted with said

35 bone precursor cell antibody to second antibody that is immunoreactive with said bone-precursor cell antibody and

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removing cells of the population that do not immunoreact
with said bone precursor cell antibody.

5   57.   The cell population of claim 56, wherein said second
antibody is conjugated to a solid substrate.

58.   The cell population of claim 57, wherein said second
10   antibody is conjugated to a plastic surface, glass
surface, agarose, polyacrylamide, or a magnetic particle.

59.   The cell population of claim 32, wherein said bone
15   precursor cells express osteocalcin, osteonectin, or bone
alkaline phosphatase but do not express the pan-
hematopoietic antigen CD34.

20   60.   The cell population of claim 32, wherein said bone
precursor cells are osteoprogenitor cells.

61.   The cell population of claim 32, wherein said bone
25   precursor cells are preosteoblast cells.

62.   A composition comprising a population of bone
precursor cells, wherein said population of bone
30   precursor cells includes cells that have the following
characteristics:

being immunoreactive with a bone precursor cell
antibody;

35

having an average cell diameter of between about 8
microns and about 70 microns; and

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differentiating into osteoblasts upon exposure to
    transforming growth factor ß, 1,25-OH Vitamin
    D3, basic fibroblast growth factor, or bone
    morphogenic protein.

5

63.   The composition of claim 62, wherein said bone
precursor cells are obtained from bone marrow cells.

10

64.   The composition of claim 62, wherein said bone
precursor cells are obtained from peripheral blood cells.

15   65.   The composition of claim 62, wherein said bone
precursor cells are mammalian bone precursor cells.

66.   The composition of claim 65, wherein said bone
20   precursor cells are human bone precursor cells.

67.   The composition of claim 62, wherein said bone
precursor cells are immunoreactive with an anti-
25   osteocalcin or anti-osteonectin antibody.

68.   The composition of claim 67, wherein said bone
precursor cells are immunoreactive with an anti-
30   osteocalcin antibody.

69.   The composition of claim 67, wherein said bone
precursor cells are immunoreactive with an anti-
35   osteonectin antibody.

70. The composition of claim 67, wherein said bone precursor cells are immunoreactive with an anti-osteocalcin and an anti-osteonectin antibody.

5

71. The composition of claim 62, wherein said bone precursor cells express osteocalcin, osteonectin, or bone alkaline phosphatase but do not express the pan-hematopoietic antigen CD34.

10

72. The composition of claim 62, wherein said bone precursor cells are osteoprogenitor cells.

15

73. The composition of claim 62, wherein said bone precursor cells are preosteoblast cells.

20   74. A method of differentiating bone precursor cells into osteoblasts, comprising the steps of:

obtaining a population of bone precursor cells in accordance with claim 32;

25

exposing said bone precursor cells to a growth factor; and

cultivating said bone precursor cell under serum
30   free conditions to differentiate said bone precursor cells into osteoblasts.

75. The method of claim 74, wherein said bone precursor
35   cells are exposed to transforming growth factor ß, 1,25-OH Vitamin D3, basic fibroblast growth factor, or bone morphogenic protein.

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76. The method of claim 75, wherein said bone precursor cells are exposed to transforming growth factor ß.

5   77. The method of claim 75, wherein said bone precursor cells are exposed to 1,25-OH Vitamin D3.

78. The method of claim 75, wherein said bone precursor
10   cells are exposed to basic fibroblast growth factor.

79. The method of claim 75, wherein said bone precursor cells are exposed to bone morphogenic protein.
15

80. The method of claim 74, wherein said bone precursor cells are mammalian bone precursor cells.

20
81. The method of claim 74, wherein said bone precursor cells are human bone precursor cells.

25   82. The method of claim 74, further comprising cultivating said bone precursor cells in the presence of type I collagen, fibrinogen, fibrin, polyglycolic acid, osteocalcin, or osteonectin.

30
83. The method of claim 82, wherein said bone precursor cells are cultivated in the presence of type I collagen.

35   84. The method of claim 82, wherein said bone precursor cells are cultivated in the presence of fibrinogen or fibrin.

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85. The method of claim 82, wherein said bone precursor cells are cultivated in the presence of polyglycolic acid or polylactic acid.

5

86. The method of claim 82, wherein said bone precursor cells are cultivated in the presence of polyglycolic acid and polylactic acid.

10

87. The method of claim 82, wherein said bone precursor cells are cultivated in the presence of type I collagen, fibrinogen, and fibrin.

15

88. The method of claim 82, wherein said bone precursor cells cultivated in the presence of type I collagen, fibrinogen, fibrin, osteocalcin, and osteonectin.

20

89. A method for identifying a subject at risk of developing an age-related bone disorder, comprising the steps of:

25   obtaining a population of cells from said subject, the population being enriched for human bone precursor cells; and

         quantifying the amount of osteocalcin or osteonectin
30   expressed by said bone precursor cells, wherein an increased amount of osteocalcin or osteonectin, in comparison to the amount within the bone precursor cells of a young or middle-aged subject, is indicative of a subject at
35   risk of developing an age-related bone disorder.

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90. A method of diagnosing a sub-set of elderly subjects with osteoporosis, comprising the steps of:

5
obtaining a population of cells from said elderly subject, the population being enriched for human bone precursor cells; and

10
quantifying the amount of osteocalcin or osteonectin expressed by said bone precursor cells, wherein a decreased amount of osteocalcin or osteonectin, in comparison to the average amount within the bone precursor cells of an elderly subject, is indicative of an elderly subject having one type of osteoporosis.

15

91. The method of claim 89 or 90, wherein said population of cells is obtained from a bone marrow sample of said patient.

20

92. The method of claim 89 or 90, wherein said population of cells is obtained from a peripheral blood sample of said patient.

25

93. The method of claim 89 or 90, wherein said population of cells is enriched for human bone precursor cells by a method including the steps of equilibrium-

30
density centrifugation separation and plastic adherence separation.

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94.   The method of claim 93, wherein said population of
cells is enriched for human bone precursor cells by a
method including the steps of equilibrium-density
centrifugation, plastic adherence and separation

5   according to cell size.

95.   The method of claim 93, wherein said population of
cells is enriched for human bone precursor cells by a

10   method including the steps of equilibrium-density
centrifugation, plastic adherence and immunomagnetic
separation.

15   96.   The method of claim 89 or 90, comprising quantifying
the amount of osteocalcin.

97.   The method of claim 89 or 90, comprising quantifying

20   the amount of osteonectin.

98.   The method of claim 89 or 90, comprising quantifying
the amount of osteonectin and the amount of osteocalcin.

25

99.   The method of claim 89 or 90, wherein quantifying
the amount of osteocalcin or osteonectin is achieved by
contacting said population of cells with an anti-

30   osteocalcin antibody or anti-osteonectin antibody and
subjecting the resultant cell population to fluorescence
activated flow cytometry.

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100. The method of claim 89, wherein an increased amount
of osteocalcin or osteonectin, in comparison to the
amount within the bone precursor cells of a young or
middle-aged subject, is indicative of an elderly subject
5   having osteoporosis.

101. The method of claim 90, wherein a decreased amount
of osteocalcin or osteonectin, in comparison to the
10   average amount within the bone precursor cells of an
elderly subject, is indicative of an elderly subject
having a more severe form of osteoporosis.

FIG. 1A

FIG. 1B

FIG. 1C

FIG. 1D
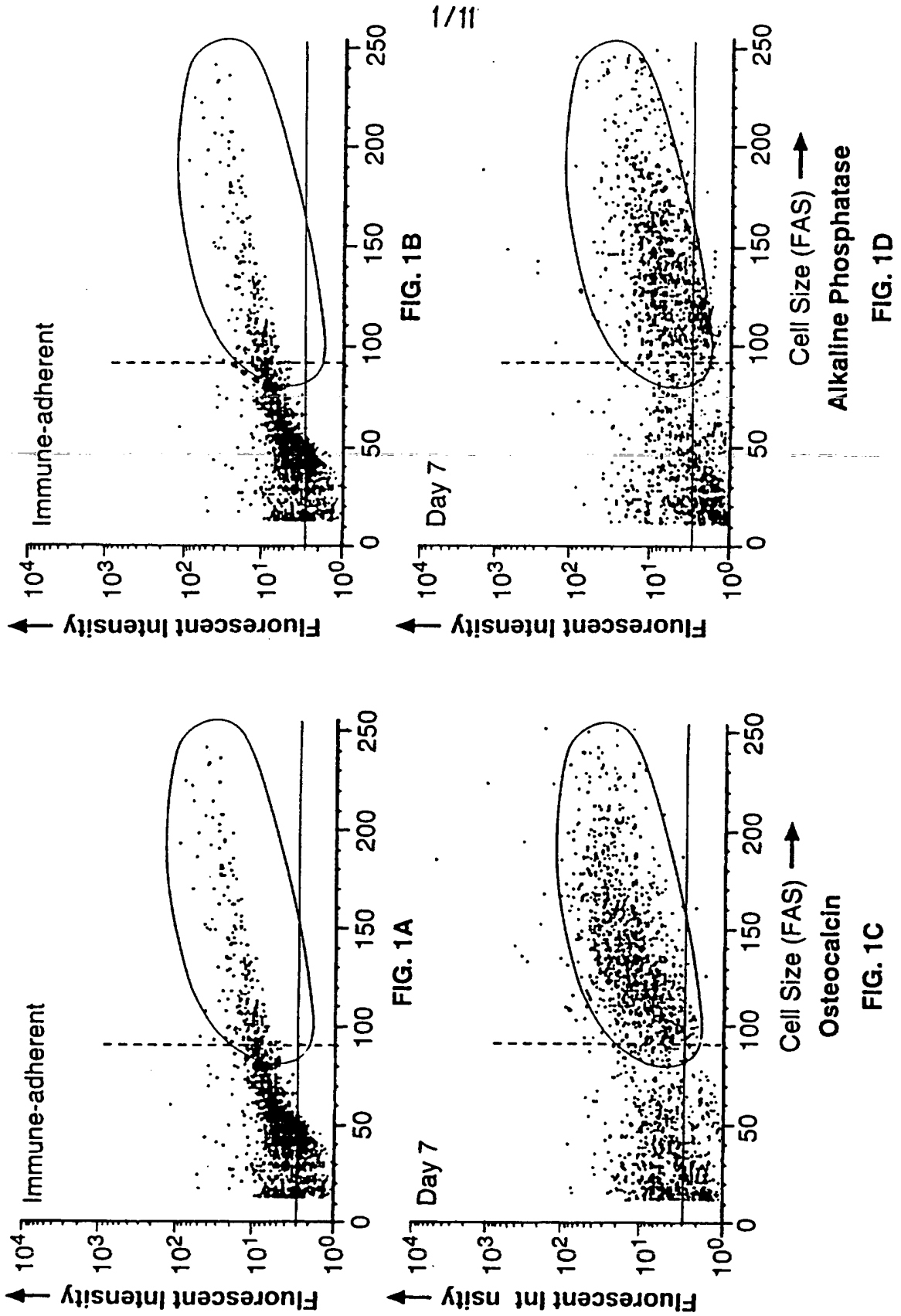
FIG. 2A
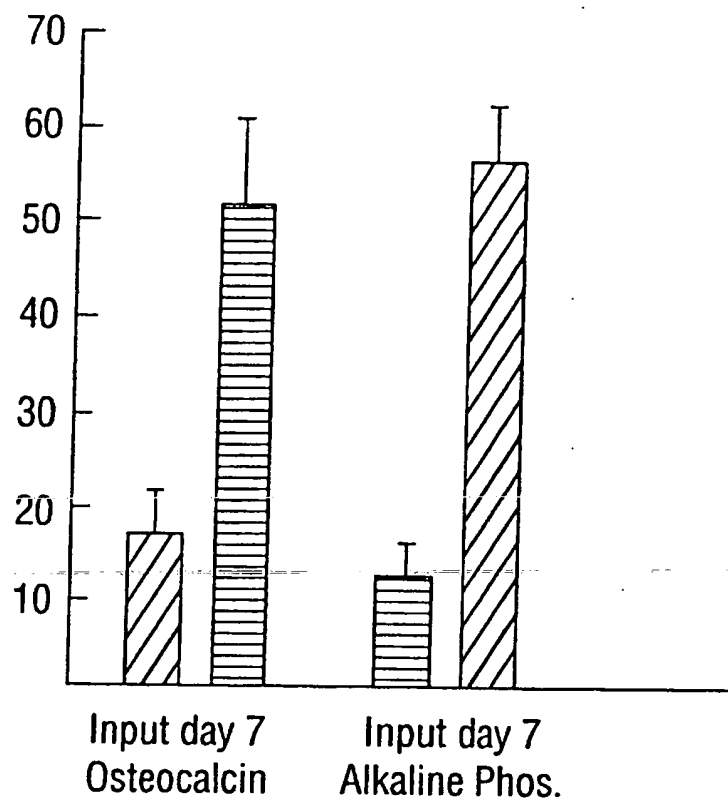
FIG. 2B
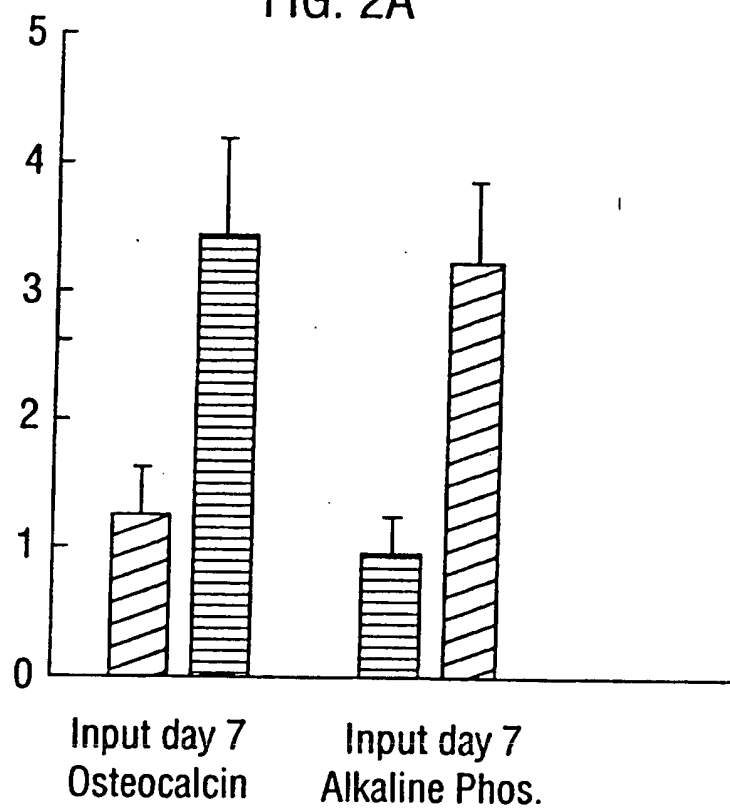
FIG.3B

FIG.3A

FIG. 4A

FIG. 4B   SUBSTITUTE SHEET (RULE 26)
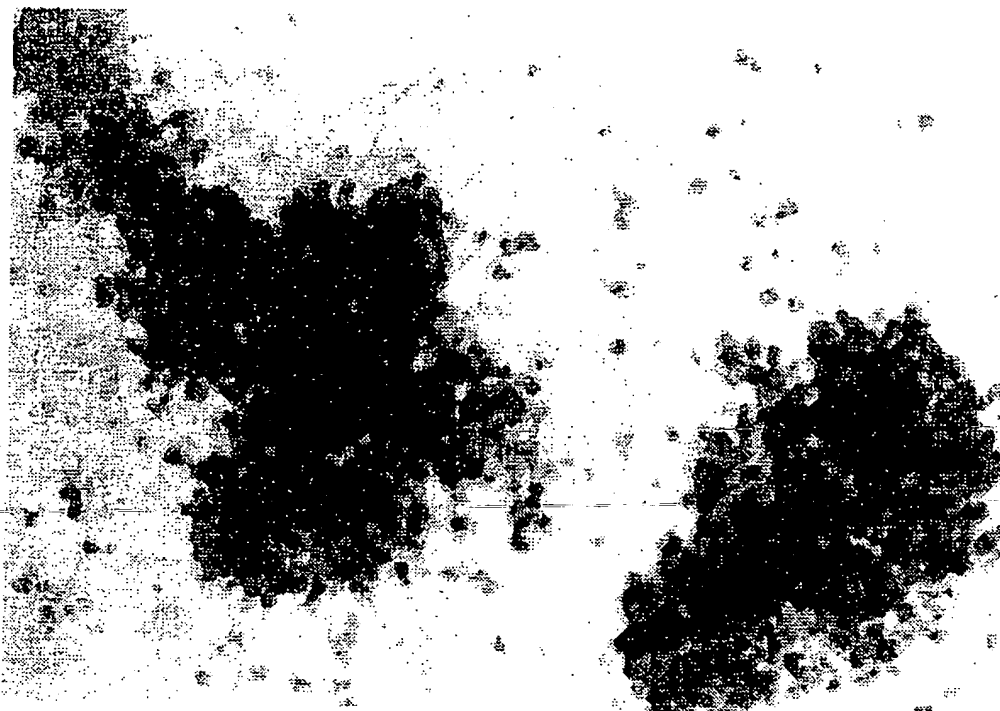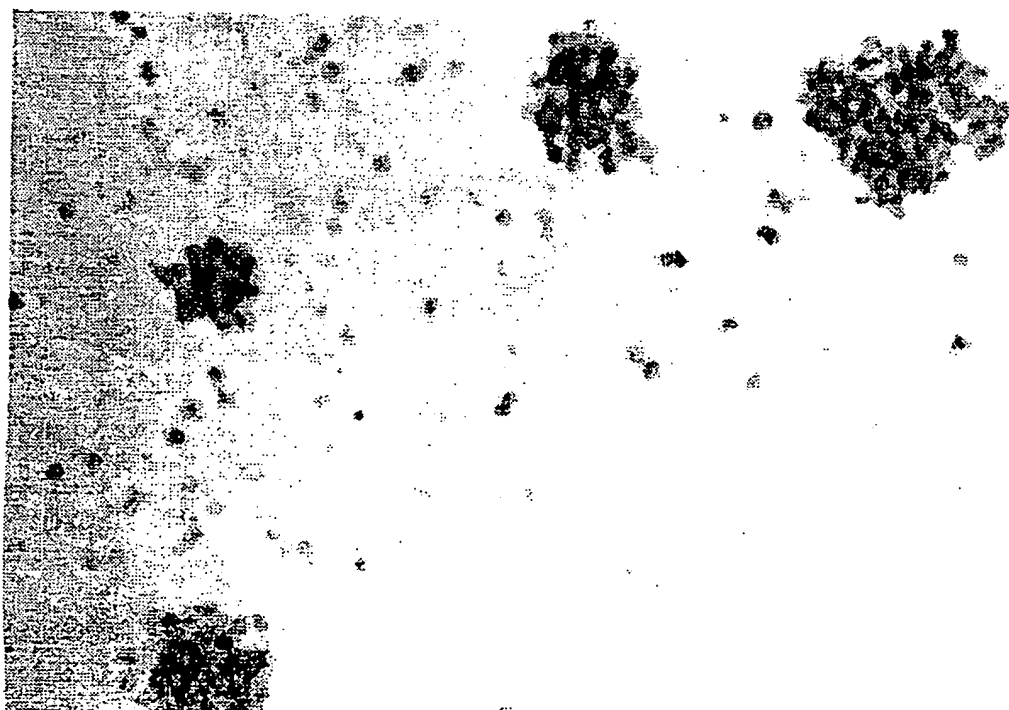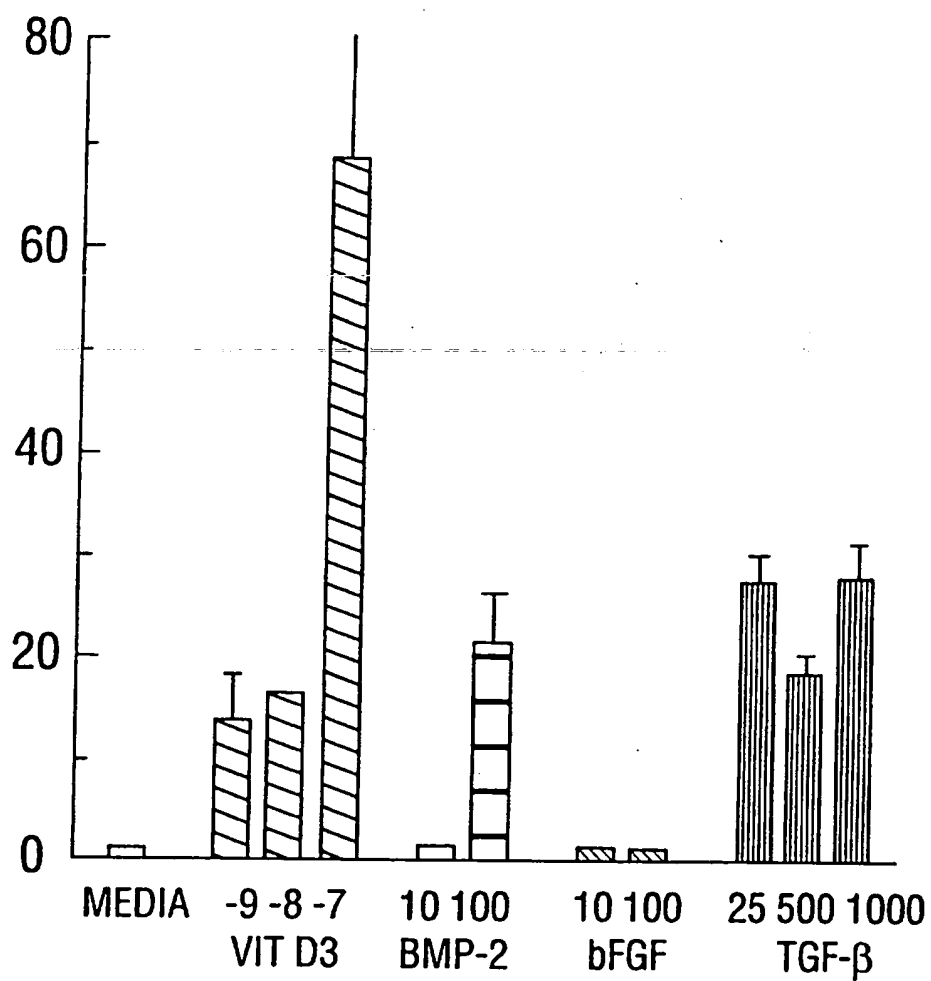
Day 0   Day 7

FIG.5A

FIG.5B

FIG.5C

FIG. 6B

Osteonectin Expression

Osteocalcin (BGP) Expression

FIG. 6A

Osteonectin Expression

Osteocalcin (BGP) Expression

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FIG. 7A

Osteocalcin (Arbitrary Fluorescence Units)

FIG. 7B

Osteonectin (Arbitrary Fluorescence Units)
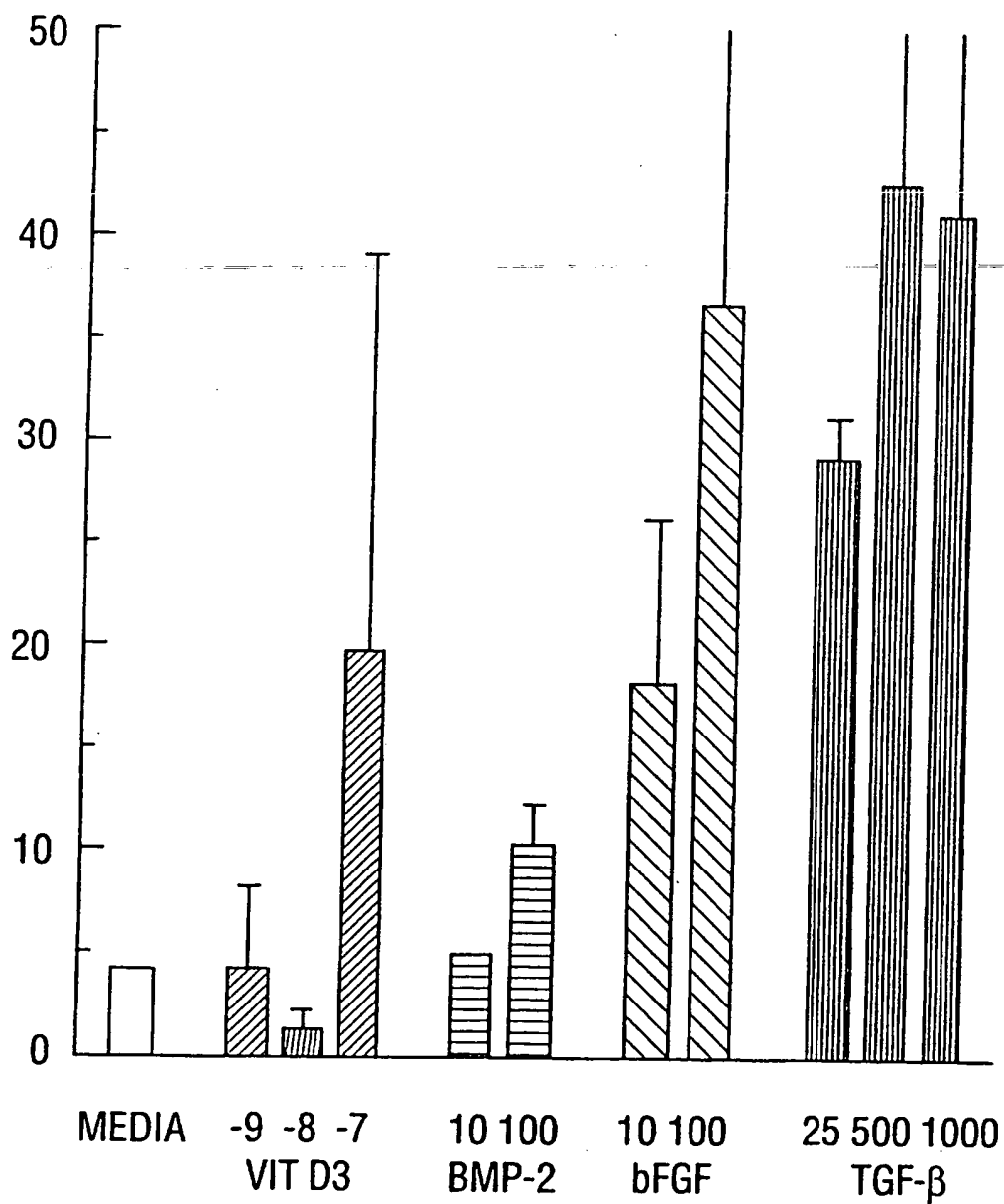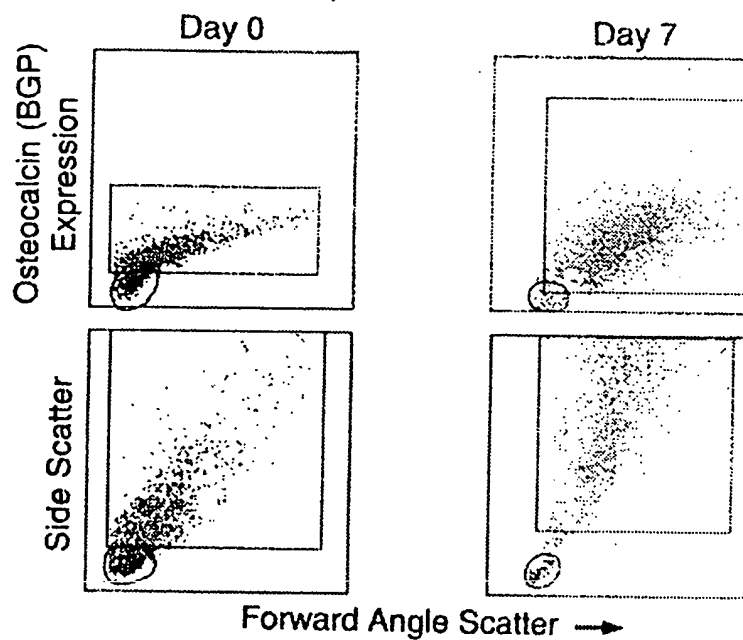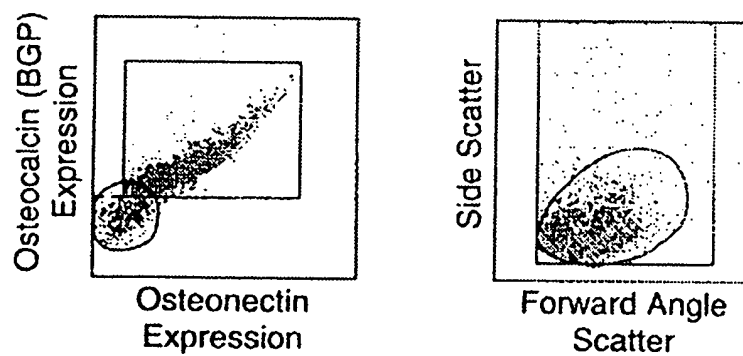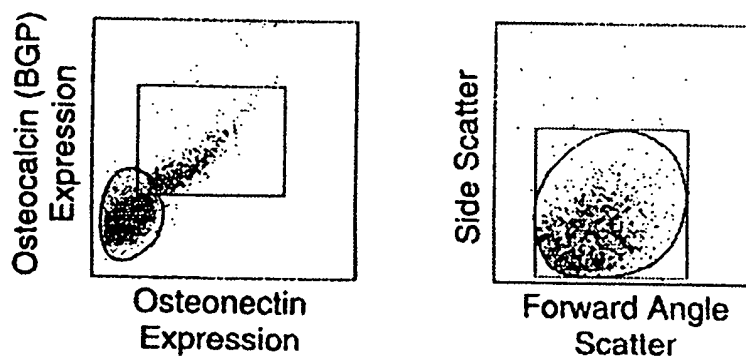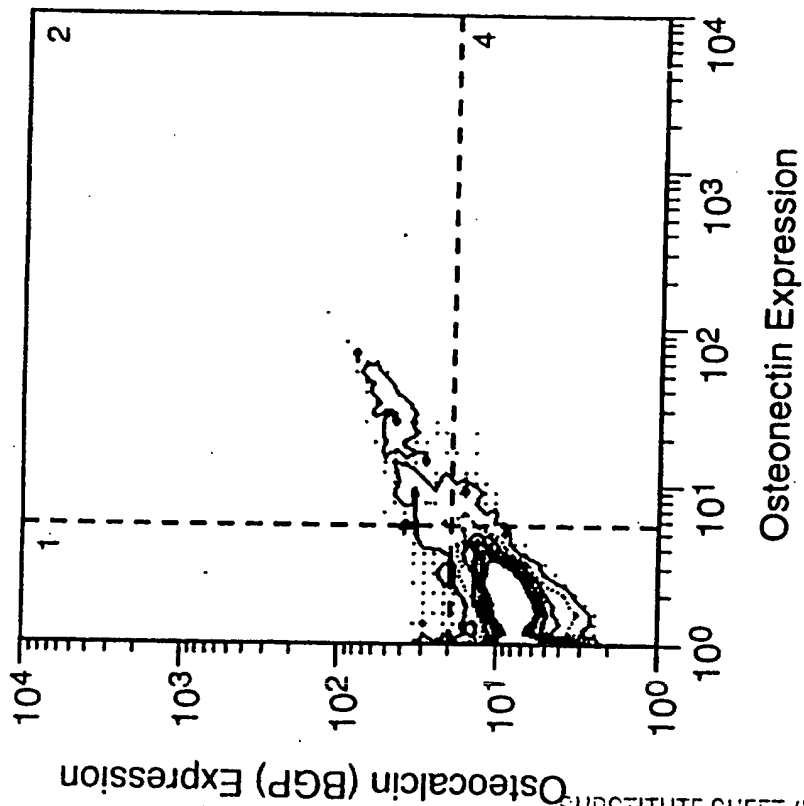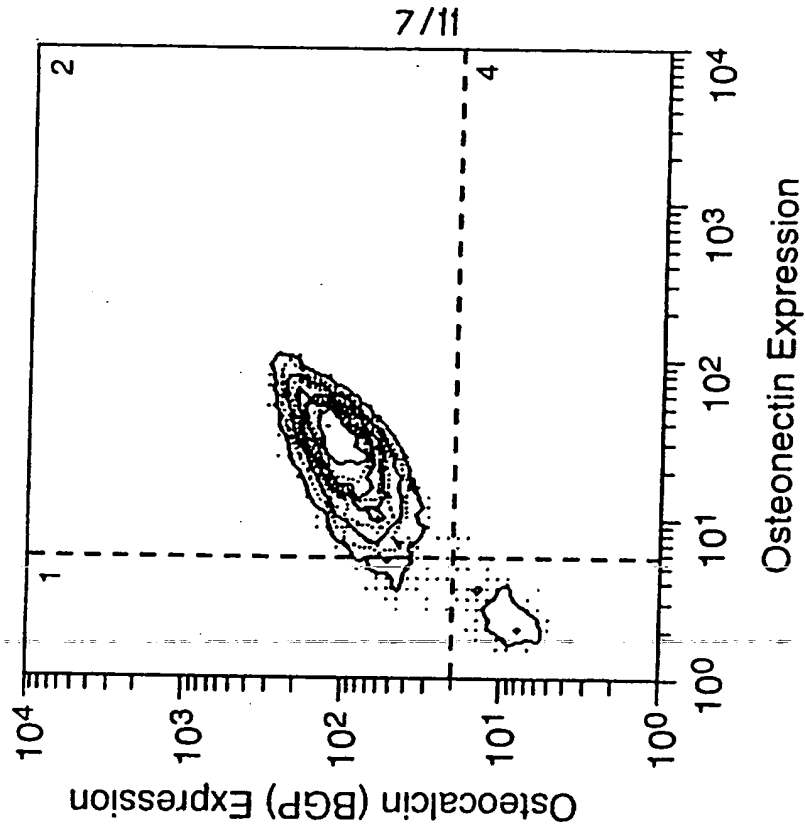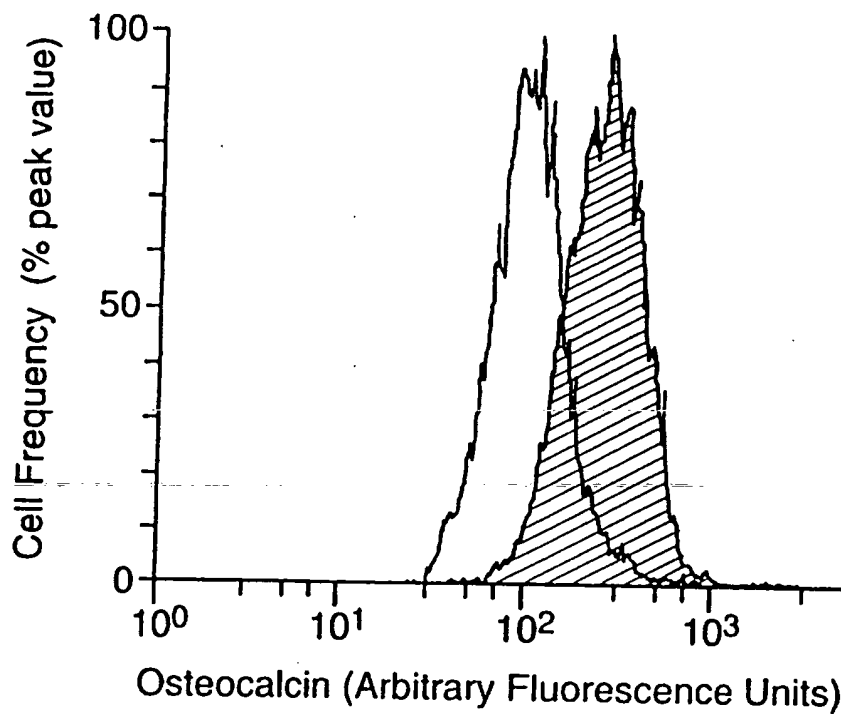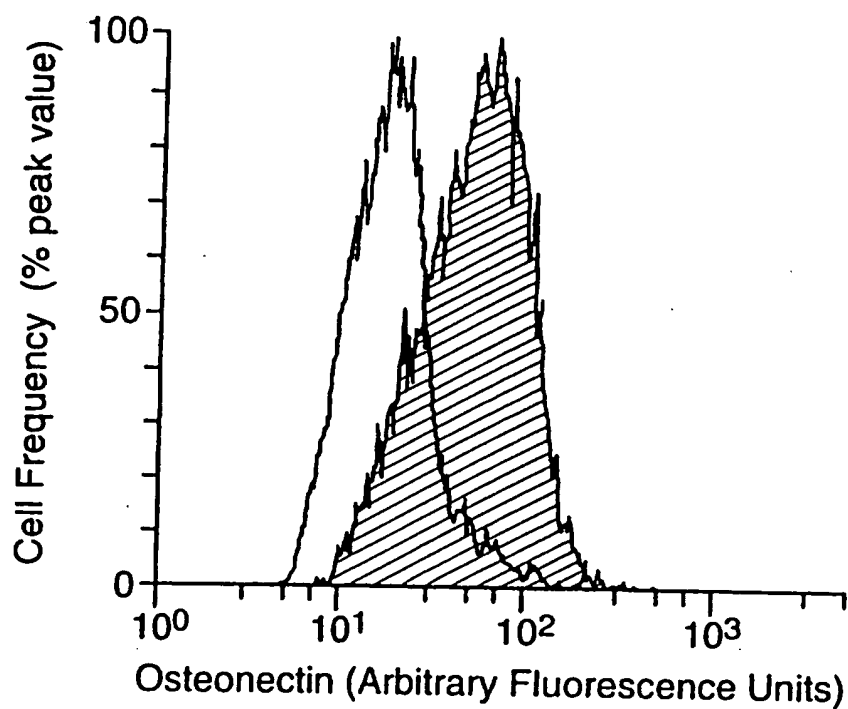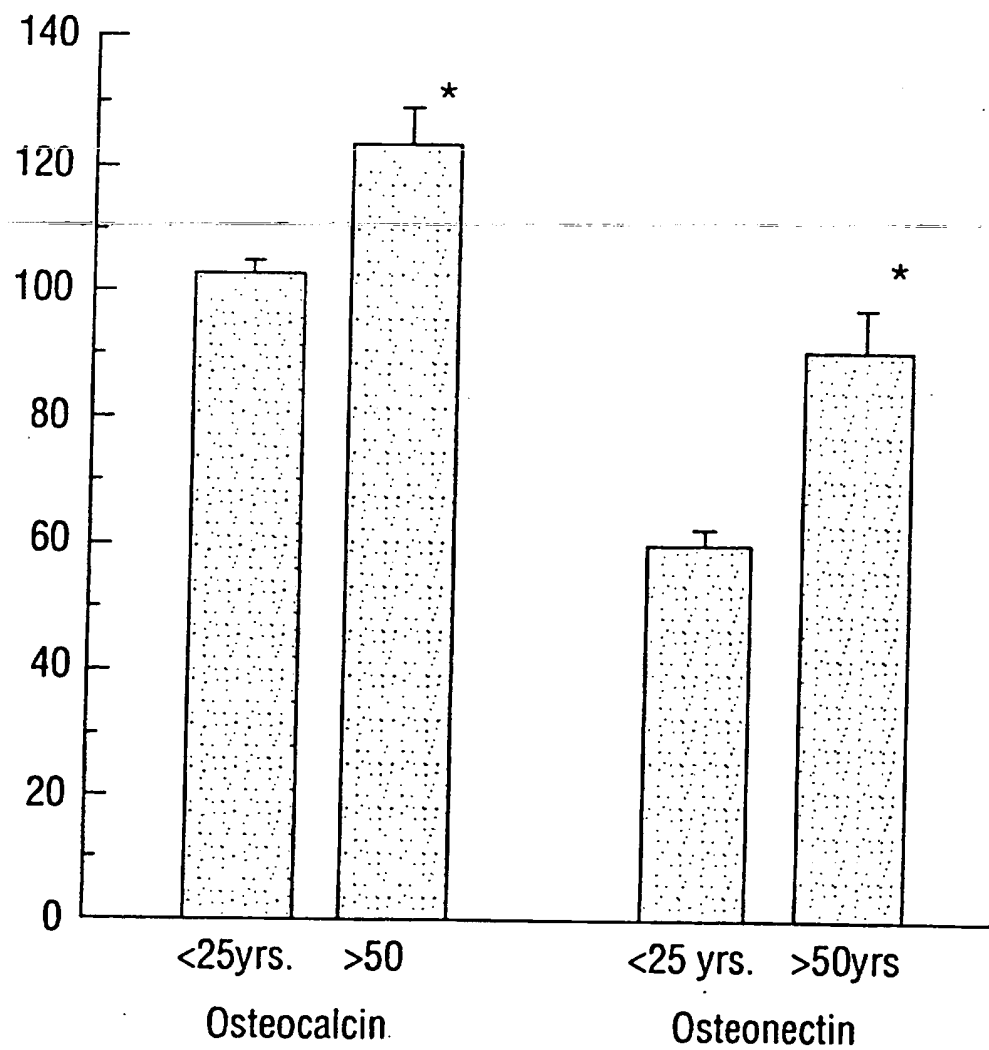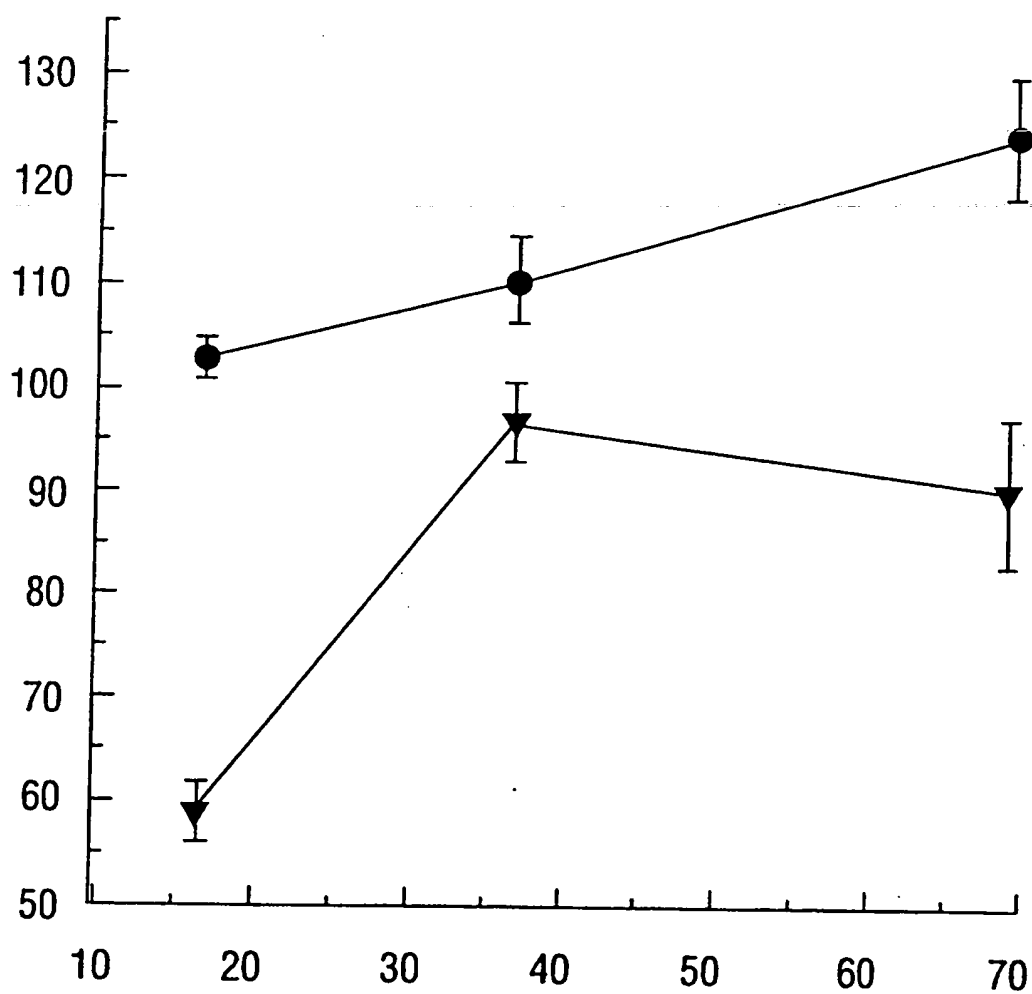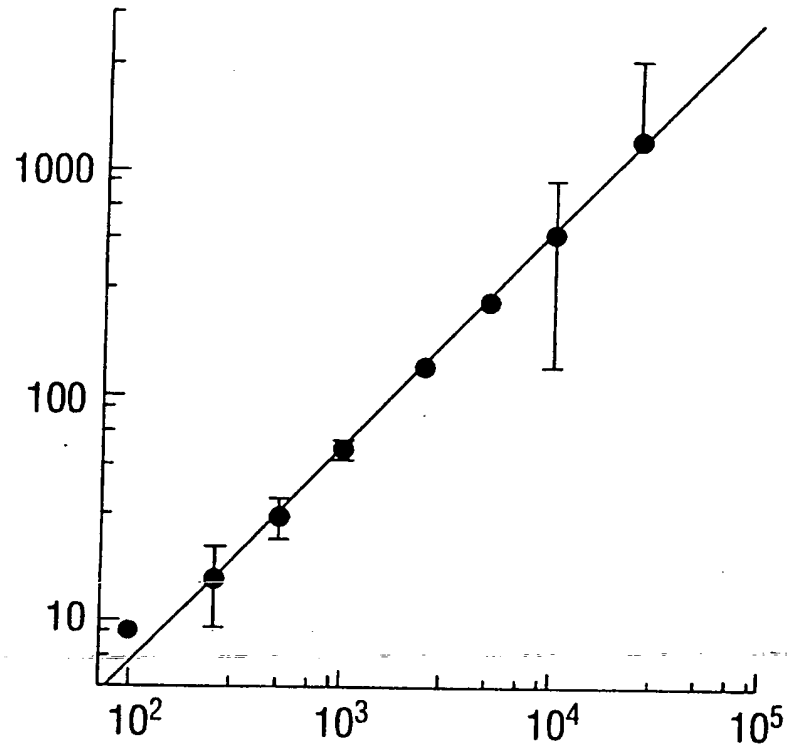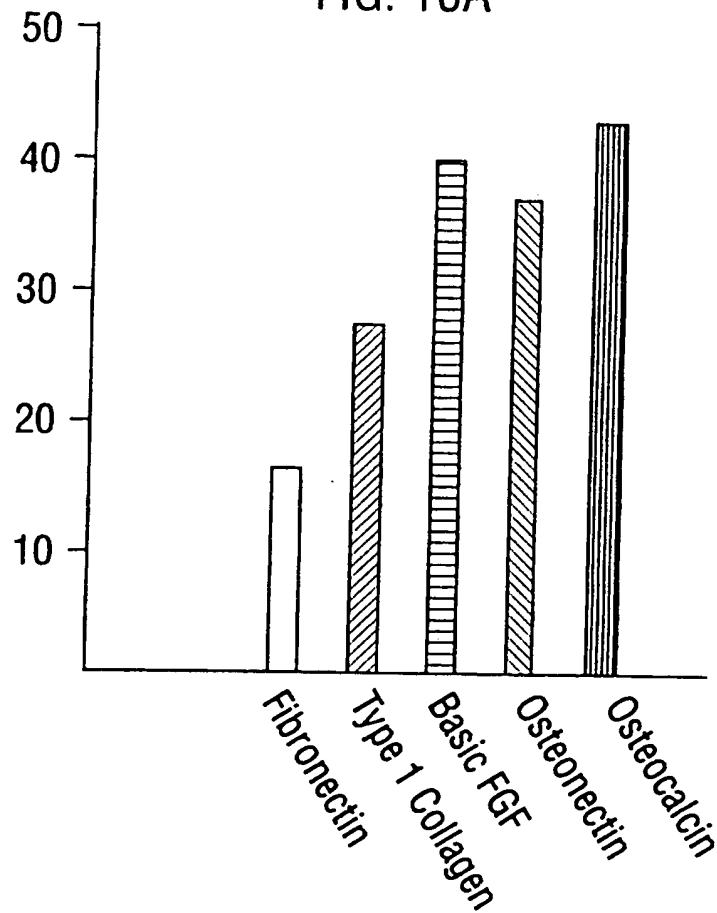
FIG. 8   SUBSTITUTE SHEET (RULE 26)

FIG. 9 SUBSTITUTE SHEET (RULE 26)

FIG. 10A

FIG. 10B

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N5/08   G01N33/569   A61K35/14   A61K35/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE MEDLINE FILE SERVER STN KARLSRUHE ABSTRACT 90105096, BRUDER ET AL 'FIRST BONE FORMATION AND THE DISSECTION OF AN OSTEOGENIC LINEAGE IN THE EMBRYONIC CHICK TIBIA IS REVEALED BY MONOCLONAL ANTIBODIES AGAINST OSTEOBLASTS'	1-16, 21-46, 51-61
Y	& BONE, (1989) 10 (5) 359-75 see abstract --- -/--	74-88,95

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 December 1995	2 9. 12. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sitch, W

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE MEDLINE FILE SERVER STN KARLSRUHE ABSTRACT 92372972, TURKSEN ET AL 'ISOLATION OF MONOCLONAL ANTIBODIES RECOGNISING RAT BONE-ASSOCIATED MOLECULES IN VITRO AND IN VIVO'	1-16, 21-46, 51-61
Y	& J HISTOCHEM CYTOCHEM, (1992 SEP) 40 (9) 1339-52 see abstract ---	74-88,95
X	DATABASE MEDLINE FILE SERVER STN KARLSRUHE ABSTRACT 93329290, CHIBA ET AL 'IMMUNOHISTOCHEMICAL LOCALIZATION OF BONE GLA PROTEIN AND OSTEONECTIN IN NORMAL HUMAN BONE AND CARTILAGE TISSUES,AND IN OSTEOSARCOMAS AND CHONDROSARCOMAS'	1-19, 21-61, 89-101
Y	& NIPPON SEIKEIGEKA GAKKAI ZASSHI, (1993 MAY) 67 (5) 463-72 see abstract ---	74-88, 95,99
X	DATABASE MEDLINE FILE SERVER STN KARLSRUHE ABSTRACT 94171900, MALAVAL ET AL 'CELLULAR EXPRESSION OF BONE-RELATED PROTEINS DURING IN VITRO OSTEOGENESIS IN RAT BONE MARROW STROMAL CELL CULTURES'	1-61, 89-101
Y	& J CELL PHYSIOL, (1994 MAR) 158 (3) 555-72 see abstract ---	74-88, 95,99
X	DATABASE MEDLINE FILE SERVER STN KARLSRUHE ABSTRACT 94360784, HARRIS ET AL 'EFFECTS OF TRANSFORMING GROWTH FACTOR BETA ON BONE NODULE FORMATION AND EXPRESSION OF BONE MORPHOGENETIC PROTEIN 2,OSTEOCALCIN,OSTEOPONTIN,ALKALINE PHOSPHATASE,AND TYPE I COLLAGEN MRNA IN LONG-TERM CULTURES OF FETAL RAT CALVARIAL OSTEOBLASTS'	62-68, 71-73, 89-94, 96,100, 101
Y	& J BONE MINER RES, (1994 JUN) 9 (6) 855-63 see abstract --- -/--	74-76, 79-88, 95,99

4

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE MEDLINE FILE SERVER STN KARLSRUHE ABSTRACT 93224544, ZHOU ET AL 'DIFFERENTIAL EFFECTS OF TRANSFORMING GROWTH FACTOR-BETA 1 AND BONE MORPHOGENETIC PROTEIN 4 ON GENE EXPRESSION AND DIFFERENTIATED FUNCTION OF PREOSTEOBLASTS'	62-67, 69, 71-73, 89-94, 97,100, 101
Y	& J CELL PHYSIOL, (1993 APR) 155 (1) 112-9 see abstract	74-76, 79-88, 95,99

X	DATABASE MEDLINE FILE SERVER STN KARLSRUHE ABSTRACT 91201417, YAMAGUCHI ET AL 'RECOMBINANT HUMAN BONE MORPHOGENETIC PROTEIN-2 STIMULATES OSTEOBLASTIC MATURATION AND INHIBITS MYOGENIC DIFFERENTIATION IN VITRO'	62-68, 71-73, 89-94, 96,100, 101
Y	& J CELL BIOL, (1991 MAY) 113 (3) 681-7 see abstract	74-77, 79-88, 95,99

X	DATABASE CHEMICAL ABSTRACTS FILE SERVER STN KARLSRUHE ABSTRACT 121:130874, CHOONG ET AL 'INDUCTION OF OSTEOBLAST DIFFERENTIATION BY ASCORBIC ACID,RETINOIC ACID,AND CALCITRIOL'	62-66, 72,73
Y	& FRONT.OSTEOSARCOMA RES. (1993), 331-4 see abstract ---	74,75, 77,80-88
X	CLIN.ORTHOP., vol. 263, 1991 pages 30-48, MOHAN ET AL 'BONE GROWTH FACTORS' see page 30,summary see page 31; table 1	62-66, 72,73
Y	see page 37, paragraph 4 - page 39, paragraph 4 see page 40, paragraph 2 - paragraph 6 ---	74-76, 78-88
X	J.CLIN.INVEST., vol. 86, 1990 pages 1387-1395, LONG ET AL 'EXPRESSION OF HUMAN BONE-RELATED PROTEINS IN THE HEMATOPOIETIC MICROENVIRONMENT' cited in the application see the whole document ---	1-77, 80-101
A	CATSIMPOOLAS 'METHODS OF CELL SEPARATION,VOLUME 1' 1977 , PLENUM PRESS , NEW YORK,USA see page 229 - page 249 ---	1-61

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BONE, vol. 13, 1992 pages 81-88, HAYNSEWORTH ET AL 'CHARACTERIZATION OF CELLS WITH OSTEOGENIC POTENTIAL FROM HUMAN MARROW' see the whole document ---	
A	US,A,5 226 914 (CAPLAN ARNOLD I ET AL) 13 July 1993 see the whole document ---	
P,X	J.CLIN.INVEST., vol. 95, no. 2, February 1995 pages 881-887, LONG ET AL 'REGULATION OF HUMAN BONE MARROW-DERIVED OSTEOPROGENITOR CELLS BY OSTEOGENIC GROWTH FACTORS' cited in the application see the whole document -----	1-101

4

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5226914	13-07-93	NONE	
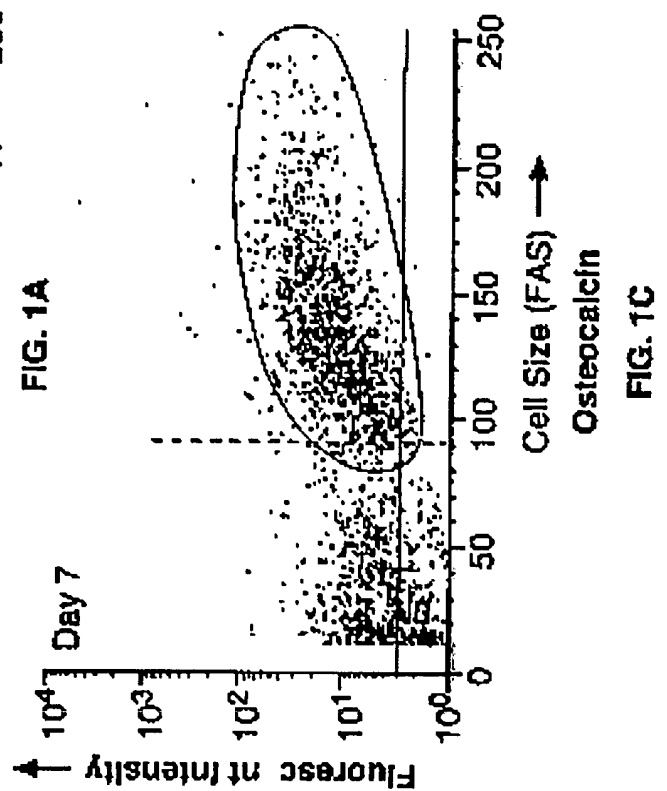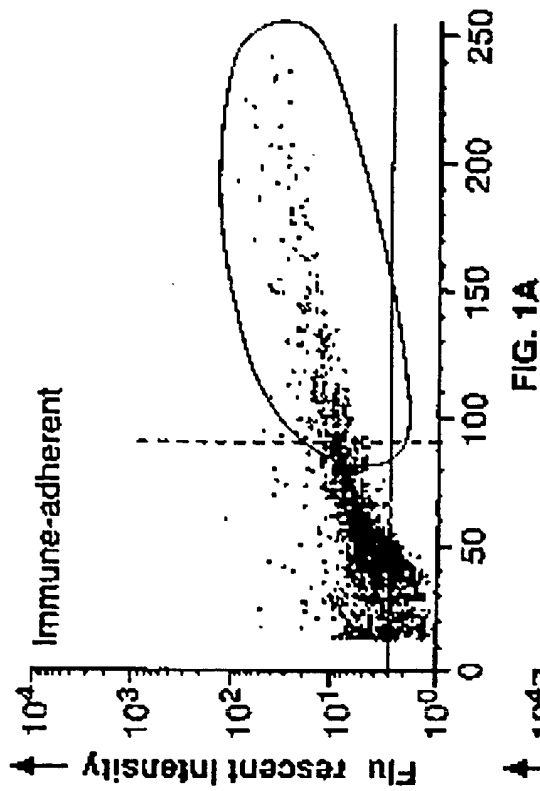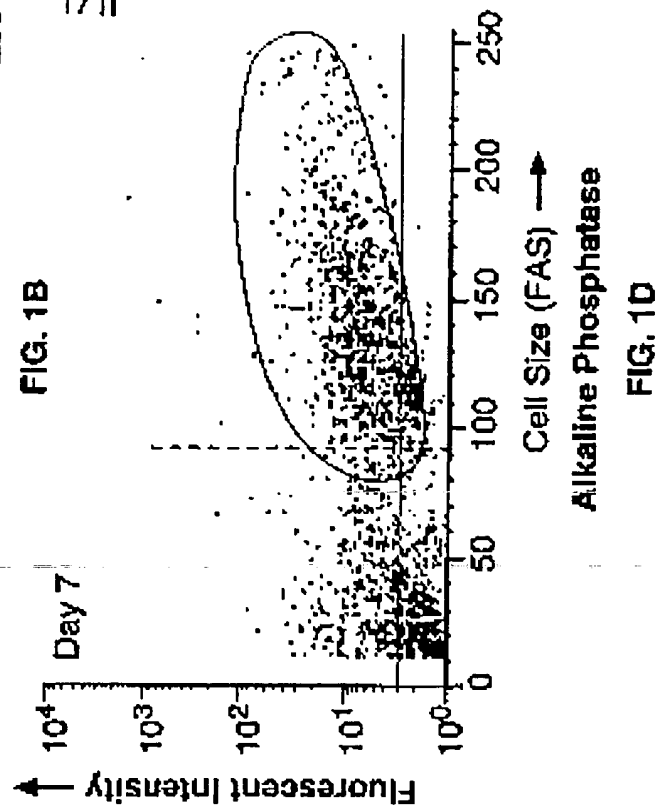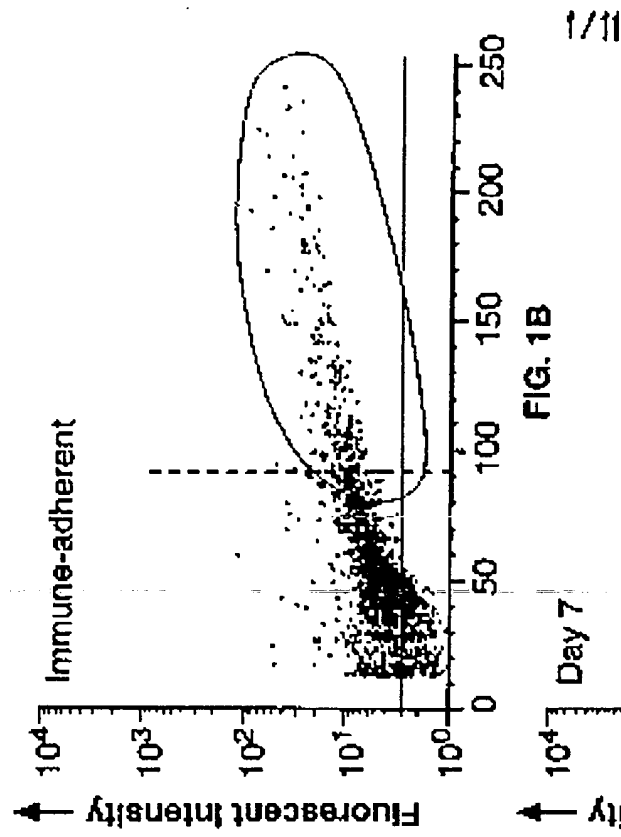
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FIG. 1A

FIG. 1B

FIG. 1C

FIG. 1D

FIG. 2A

FIG. 2B

FIG. 3B

FIG. 3A

FIG. 4A

FIG. 4B

Day 0   Day 7

Osteocalcin (EGP) Expression

Side Scatter

Forward Angle Scatter →

FIG.5A

Osteocalcin (BGP) Expression

Osteonectin Expression

Side Scatter

Forward Angle Scatter

FIG.5B

Osteocalcin (BGP) Expression

Osteonectin Expression

Side Scatter

Forward Angle Scatter
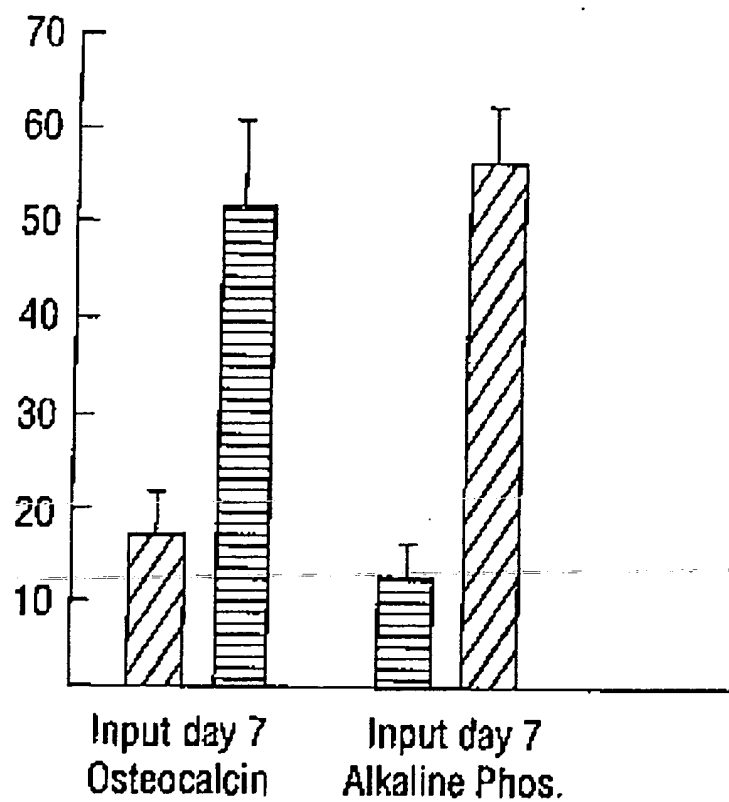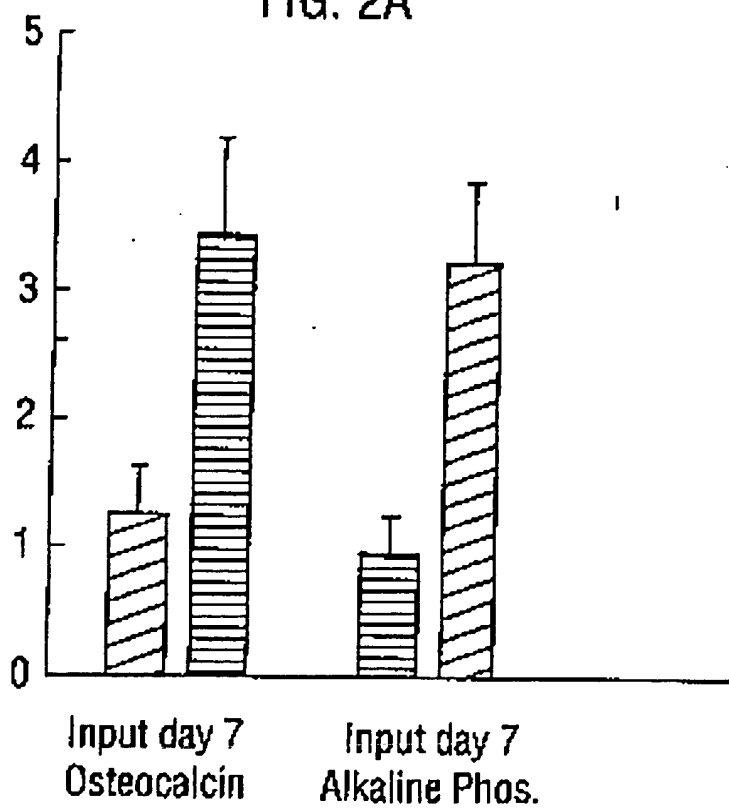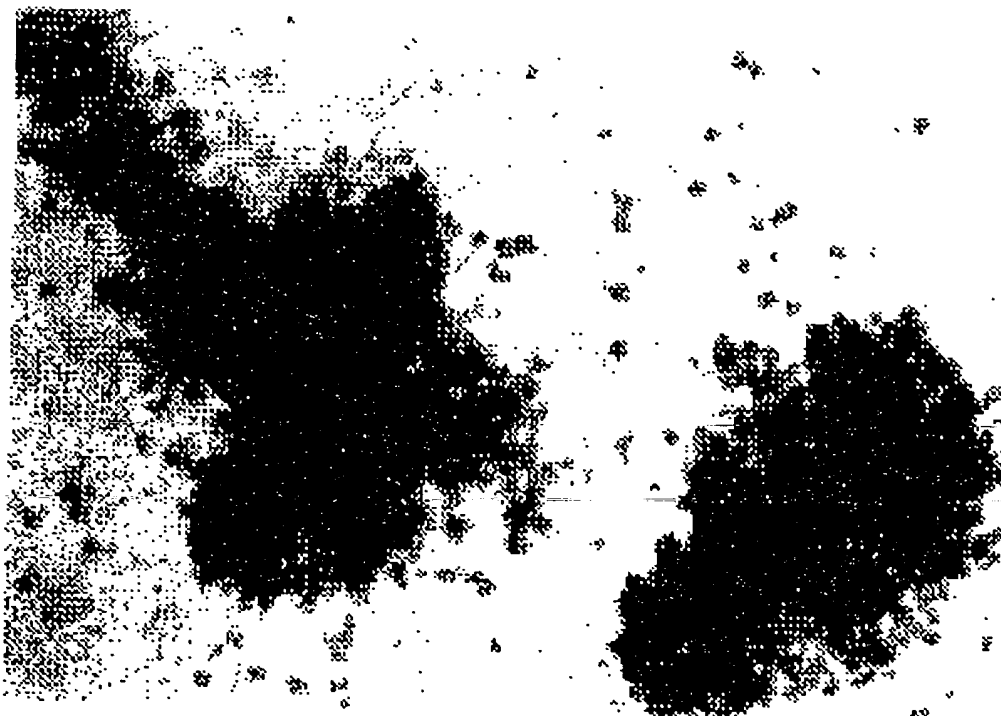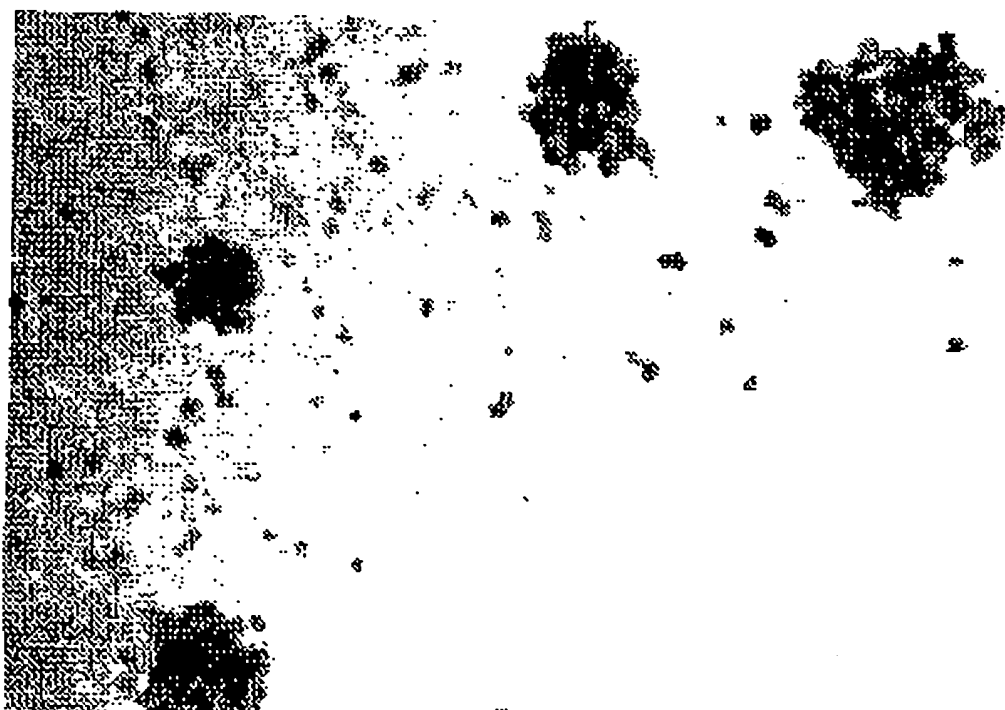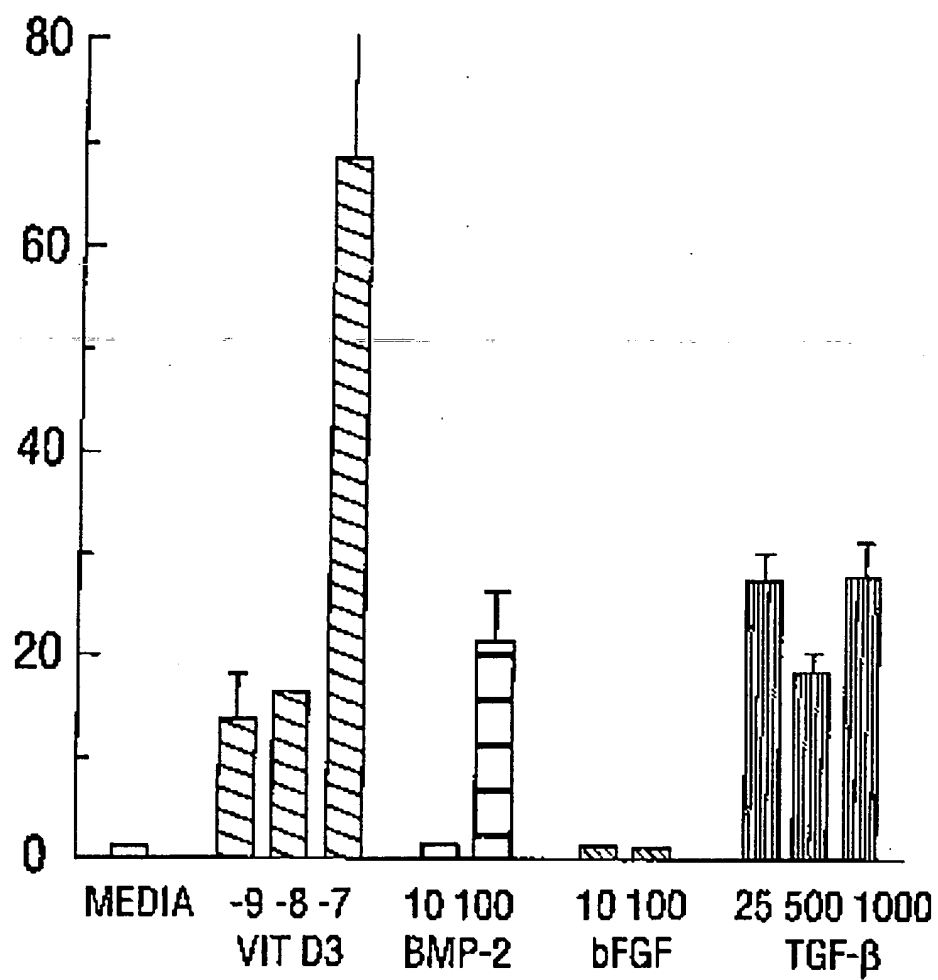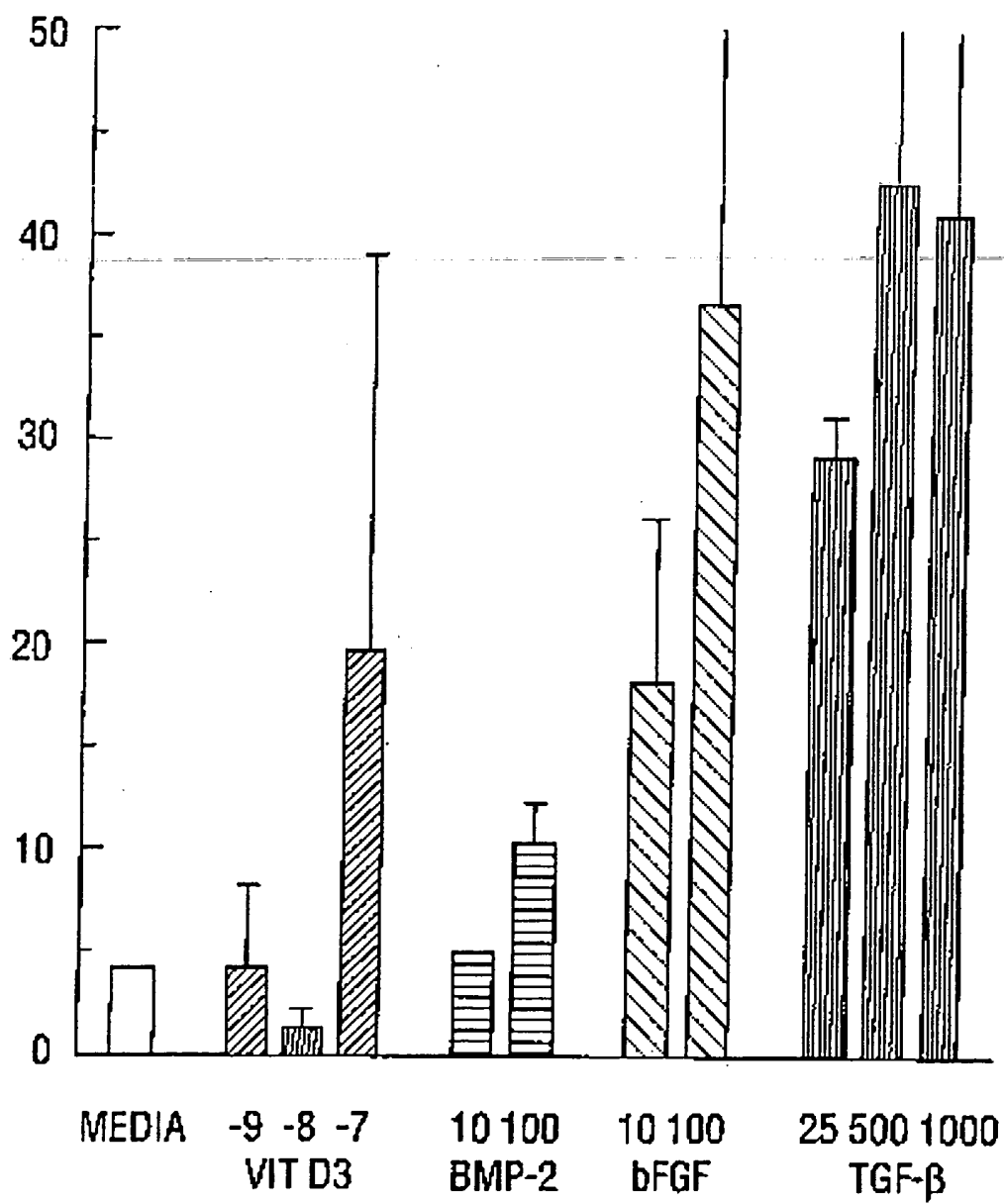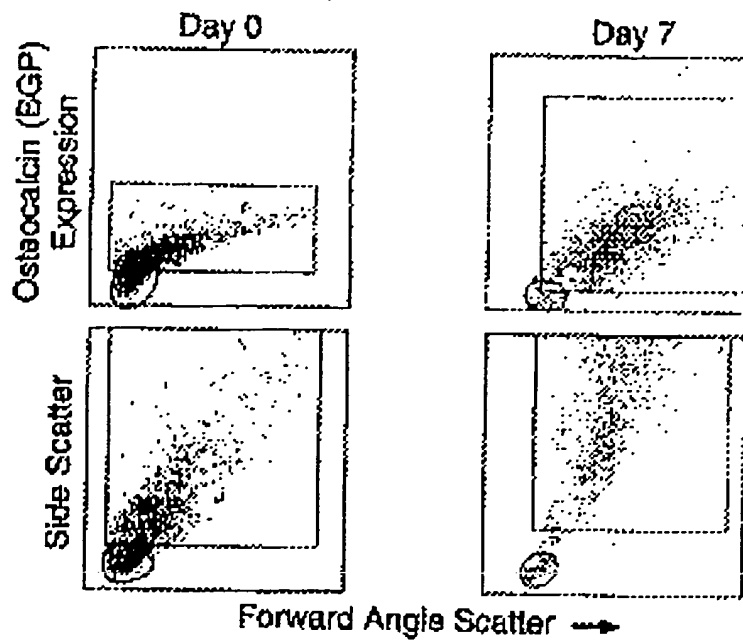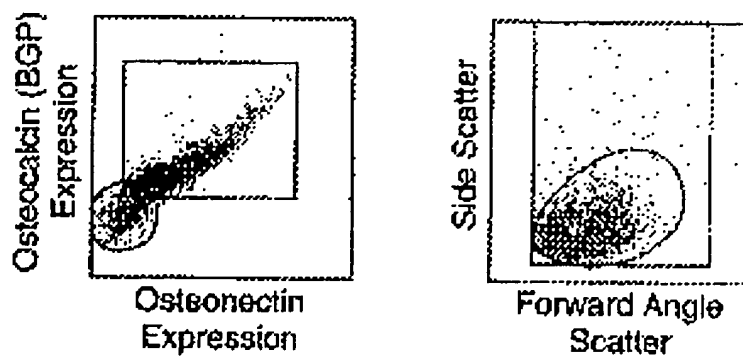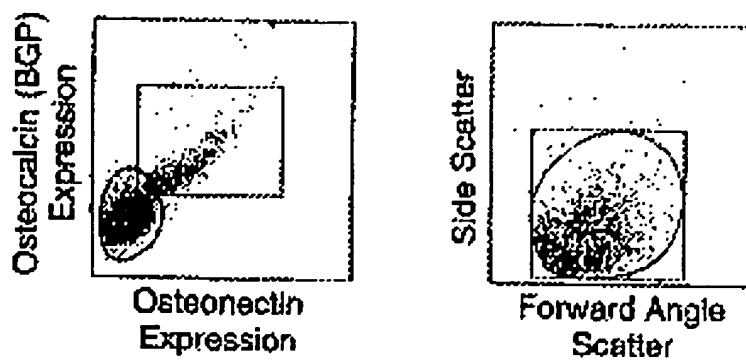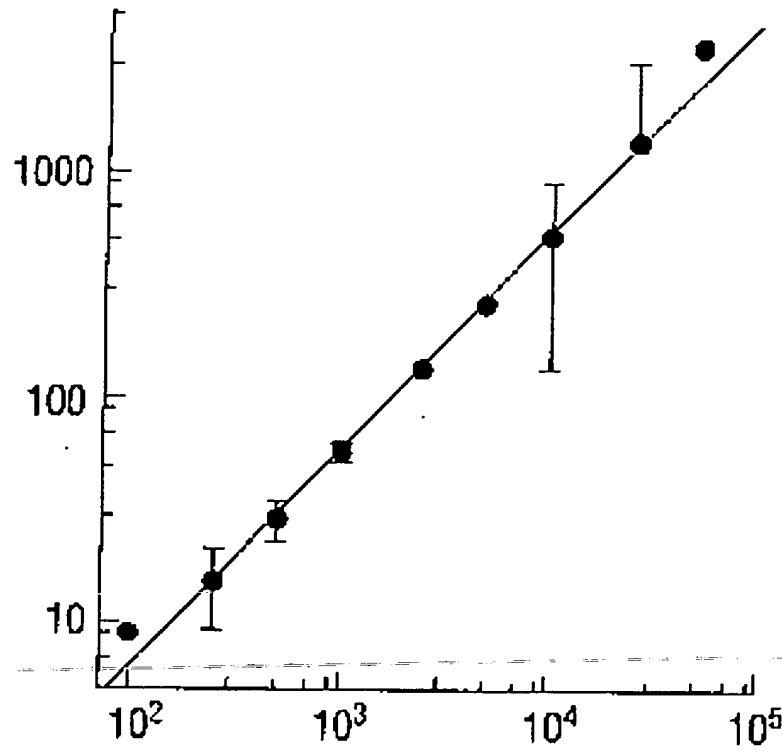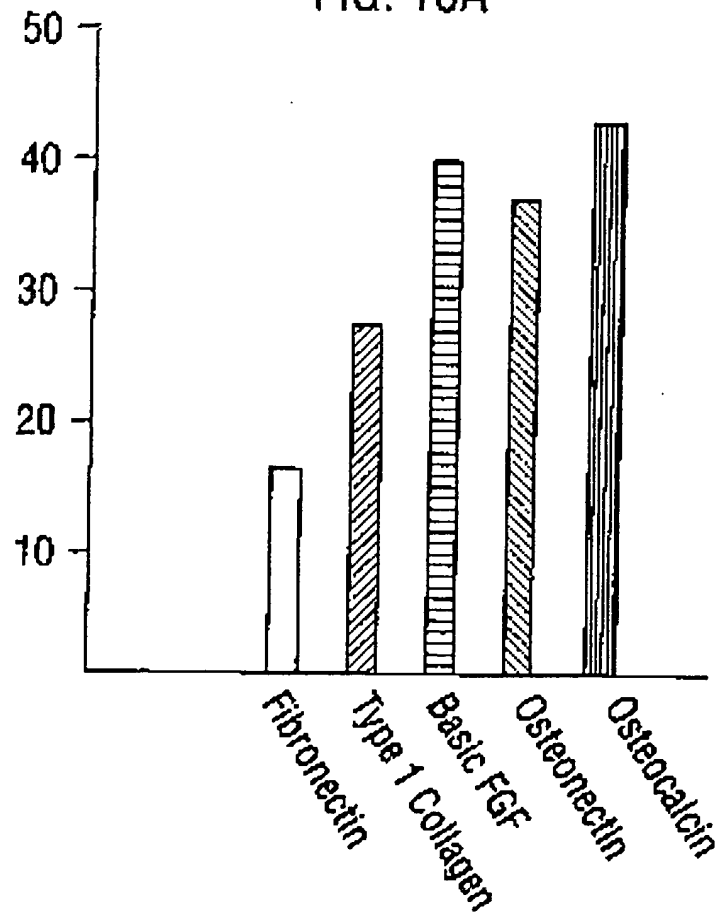
FIG.5C

SUBSTITUTE SHEET (RULE 26)

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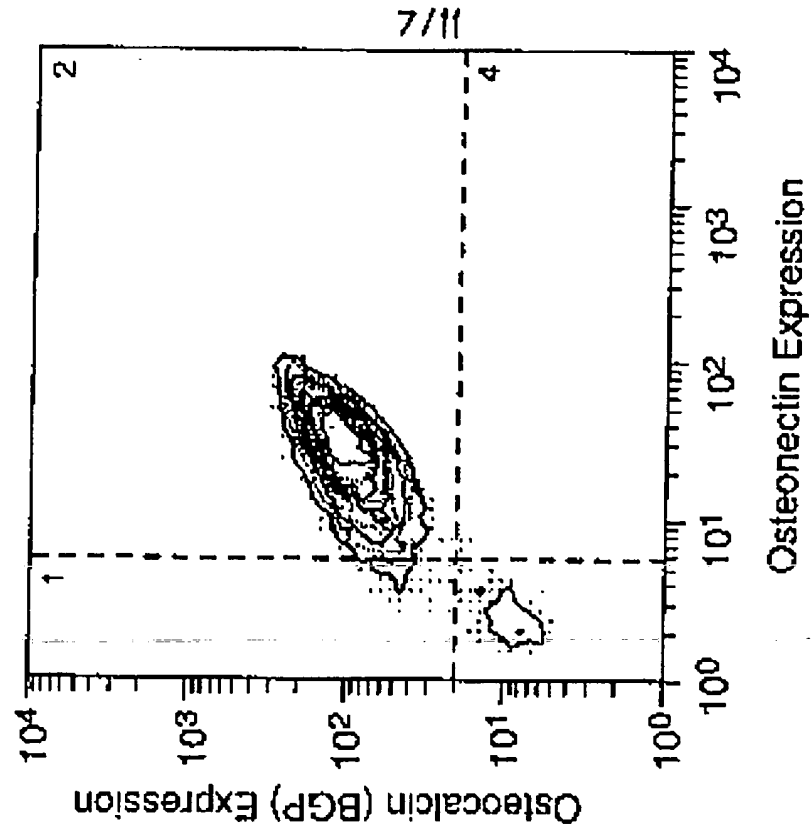
FIG. 6B

FIG. 6A

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FIG. 7A

FIG. 7B

FIG. 8

FIG. 9 SUBSTITUTE SHEET (RULE 26)

3NSDOCID: <WO___9605290A1TI_>

FIG. 10A

FIG. 10B